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Effects of Chemotherapy on Bone Growth and Chondrocyte Cell Death Signaling

Farasat Zaman

Karolinska Institutet

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Stockholm 2010

ABSTRACT

Glucocorticoids (GCs) are widely used in both children and adults to treat common inflammatory diseases, including asthma, rheumatoid arthritis, ulcerative colitis and Crohn's disease. However, a multitude of undesired side effects have been reported in patients being treated with GCs, such as osteoporosis, obesity, metabolic disturbances, myopathy and decreased linear bone growth (in children). Dexamethasone, a widely used GC, often causes bone growth impairment as an undesired side-effect in treated children. This observation is supported by experimental data showing that dexamethasone alters proliferation/differentiation and abnormally triggers apoptosis within the growth plate, which may play a key role in the pathophysiology of dexamethasone-induced growth retardation. By investigating these mechanisms, we found that dexamethasone activates caspase-8, -9 and -3 in proliferative chondrocytes. In addition, the Akt-PI3K signaling pathway, which plays a key role in the survival and proliferation of growth plate chondrocytes, is also impaired due to dexamethasone-induced inhibition of Akt phosphorylation. The observation of caspase-9 activation from these studies suggests that an intrinsic apoptotic pathway is also activated in chondrocytes. Therefore, we hypothesized that Bax, a pro-apoptotic member of the Bcl-2 family that is known to regulate intrinsic apoptosis, may play a key role in dexamethasone-induced retardation of bone growth. In chondrocytes, dexamethasone induced conformational changes in Bax, dissipation of the mitochondrial membrane potential and resulted in the release of cytochrome c. Further, Bax-siRNA prevented chondrocytes from undergoing apoptosis. Bax activation was also observed in human growth plate cartilage specimens cultured *ex vivo* in the presence of dexamethasone. Finally, we observed that Bax-deficient mice were protected from dexamethasone-induced bone growth retardation. Collectively, our data reveal a novel role for Bax in dexamethasone-induced bone growth retardation and impaired bone formation. These findings highlight the possibility for new therapeutic approaches to prevent GC-induced growth failure by specifically targeting Bax (Paper-I, II).

Proteasome inhibitors (PIs) such as MG262 and bortezomib are a novel class of anticancer drugs. Bortezomib has recently been introduced clinically to treat multiple myeloma and is under clinical trials in children to treat various cancers. Here we show for the first time that systemic administration of PIs specifically impairs the ubiquitin/proteasome system (UPS) in growth plate chondrocytes. Importantly, we found that young mice display severe growth retardation during treatment, as well as 45 days after the cessation of treatment, with clinically relevant amounts of PIs. Dysfunction of the UPS was also accompanied by the induction of apoptosis (p53-, apoptosis-inducing factor (AIF)- and Bax-mediated apoptosis) of stem-like and proliferative chondrocytes in the growth plate. We also provide evidence that AIF serves as a direct target protein for ubiquitin, thus explaining its prominent up-regulation upon proteasome inhibition. Suppression of p53 or AIF expression with siRNA partially rescued chondrocytes from PI-induced apoptosis (35 and 41%, respectively). These findings show that PIs may selectively target essential cell populations in the growth plate, causing significant growth failure, and our results could have important implications for the use of PIs in the treatment of childhood cancer (Paper-III, IV).

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Farasat Zaman

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Stockholm 2010

Cover photo
Impaired ubiquitin proteasome system (accumulation of Ub-GFP)
in the growth plate of mice.

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To all scientists

Who are working anonymously to make our life pleasant and for their efforts in understanding the laws of nature!

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LIST OF PUBLICATIONS FOR THESIS DEFENCE

I. Chrysis D, **Zaman F**, Chagin AS, Takigawa M, Sävendahl L. 2005. Dexamethasone induces apoptosis in proliferative chondrocytes through activation of caspases and suppression of the Akt-phosphatidylinositol 3'-kinase signalling pathway. *Endocrinology*; 146(3):1391-7.

II. **Zaman F**, Chrysis D, Huntjens K, Fadeel B, Sävendahl L. 2010. Absence of the pro-apoptotic Bax protein protects from glucocorticoid-induced bone growth impairment. Manuscript submitted to *Cell Death & Differentiation.*

III. **Zaman F**, Menendez-Benito V, Eriksson E, Chagin AS, Takigawa M, Fadeel B, Dantuma NP, Chrysis D, Sävendahl L. 2007. Proteasome inhibition up-regulates p53 and apoptosis-inducing factor in chondrocytes causing severe growth retardation in mice. *Cancer Res 7;* 67(20): 10078-86.

IV. Eriksson EE, **Zaman F**, Sävendahl L. Bortezomib induces apoptosis of stem-cell like chondrocytes causing growth retardation. 2010. Manuscript submitted to *Cancer Research.*

ADDITIONAL PUBLICATIONS (Not included in thesis)

1. Lindahl E, Nyman U, **Zaman F,** Palmberg C, Cascante A, Shafqat J, Takigawa M, Sävendahl L, Jörnvall H, Joseph B. 2010. Proinsulin C-peptide regulates ribosomal RNA expression. *J Biol Chem*; 285(5):3462-9.

2. **Zaman F**, Fadeel B, and Sävendahl L. 2008. Proteasome inhibition therapies in childhood cancer. *Leukemia;* 22(4):883-4.

3. Colon E, **Zaman F**, Axelsson M, Larsson O, Carlsson-Skwirut C, Svechnikov KV, Söder O. 2007. Insulin-like growth factor-I is an important anti-apoptotic factor for rat Leydig cells during postnatal development. *Endocrinology;* 148(1):128-39.

4. Chagin AS, Karimian E, **Zaman F,** Takigawa M, Chrysis D, Sävendahl L. 2007. Tamoxifen induces growth retardation in fetal rat metatarsal bones by massive apoptosis of chondrocytes in the growth plate cartilage. *Bone*; 40(5):1415-24.

5. Nurmio M, Toppari J, **Zaman F,** Andersson AM, Paranko J, Söder O, and Jahnukainen K. 2007. Inhibition of tyrosine kinases C-kit and PDGFR by imatinib mesylate interferes with postnatal testicular development in the rat. *Int J Androl;* 30(4):366-76.

CONTENTS

List of abbreviations

BaxKO	Bax knockout
ECM	Extracellular matrix
Dexa	Dexamethasone
DISC	Death-inducing signaling complex
DR	Death Receptor
FGF-2	Fibroblast growth factor-2
GC	Glucocortcoid
GCs	Glucocorticoids
GH	Growth hormone
IGF-I	Insulin-like growth factor-I
MPT	Mitochondrial membrane potential
PIs	Proteasome inhibitors
RA	Rheumatoid arthritis
TMRE	Tetramethylrhodamine ethyl ester
TUNEL	Terminal deoxynucelotidyle transferase-mediated deoxy-UTP nick-end labelling
Ub	Ubiquitin
UPS	Ubiquitin proteasome system
VEGF	Vascular endothelial growth factor
WT	Wild type

1 FOREWORD

This thesis concerns the effects of chemotherapy on bone growth in children. The overall aim was to characterize how different chemotherapeutic drugs, such as glucocorticoids and novel proteasome inhibitors (including bortezomib) affect bone growth in growing individuals. In this thesis, I used any array of experimental model systems, including cell lines (rat and human), microsurgery of fetal rat metatarsal bones (organ culture), normal and genetically modified mice models, normal rats, and biopsies of human growth plate tissues obtained from children for use as preclinical models. Finally, using the above-mentioned experimental models, an attempt has been made to identify new molecular targets/treatment strategies to prevent bone growth failure in children and determine if they can be clinically used as better treatment strategies.

Stockholm, November 2010

Farasat Zaman

2 INTRODUCTION

2.1 DRUG-INDUCED LONGITUDINAL BONE GROWTH IMPAIRMENT IN CHILDREN

Children being treated with various drugs can generally tolerate acute side effects very well, but there are reports showing that these children are also vulnerable to other side effects such as bone growth impairment. Bone, among other tissues, is indeed a frequent target of side effects caused by certain chemotherapeutic drugs. In particular, children may grow poorly long after termination of chemotherapy, suggesting irreversible damage to skeletal growth and development (Siebler, Shalet et al. 2002).

In an attempt to investigate drug-induced longitudinal bone growth impairment, we examined the effects of two groups of drugs: glucocorticoids (GCs) and novel proteasome inhibitors (PIs), such as bortezomib. We chose to characterize the effects of GCs and PIs on bone growth because they are widely used in the treatment of various diseases, including asthma, inflammatory bowel disease and rheumatoid arthritis. GCs are administered alone and in combination with other drugs. Unfortunately, the wide and frequent use of GCs to treat multiple diseases is associated with short stature and osteoporosis, which are important long-term side effects in the treated patients. Similarly, the identification of promising molecular targets to treat diseases, including cancer, has led to the development of many exciting new drugs, such as the PIs bortezomib and MG262. We and others recently reported that PI treatment in young, fast-growing individuals induces severe growth retardation (Zaman, Menendez-Benito et al. 2007). Bortezomib has recently been introduced in the clinic to treat multiple myeloma and is currently in clinical trials to treat childhood cancers (Blaney, Bernstein et al. 2004). Therefore, it is important to further investigate the effects of PIs on bone growth.

The following sections present basic information on longitudinal bone growth, chemotherapy and cell death signaling, which is necessary to understand the effects of GCs and PIs on bone growth.

2.2 LONGITUDINAL BONE GROWTH

The process of bone elongation/longitudinal bone growth is complex and tightly regulated by several factors, such as nutritional, neuronal and hormonal mechanisms, which are all necessary for optimal bone growth. Indeed, any imbalance in these factors may result in impaired bone growth. Longitudinal bone growth takes place in the growth plates. Growth plates, also known as the physis, are areas (i.e., thin layers) of developing cartilage tissue near the end of the long bones in children and adolescents. These growth plates consist of a highly organized population of cells called chondrocytes, which form three distinct layers: the resting, proliferative, and hypertrophic zones **(Figure 1)**.

2.2.1 Role of Resting, Proliferative and Hypertrophic Chondrocytes in Longitudinal Bone Growth

In the resting zone, chondrocytes are irregularly scattered in the cartilage matrix. These resting chondrocytes, also known as stem-like cells, mainly function as a reservoir for the growth plate and give rise to proliferative and hypertrophic chondrocytes (Hunziker 1994) (Abad, Meyers et al. 2002). The resting zone chondrocytes are round, small and exhibit a low proliferative rate. In rabbits, it has been shown that the presence of only the resting zone in the growth plate is enough to re-establish the entire growth plate. In these studies, both the proliferative and hypertrophic zones were removed to determine if resting zone chondrocytes can behave like stem-like cells in the growth plate (Abad, Meyers et al. 2002). These studies revealed that resting/stem-like cells are of prime importance for the growth plate, and any disturbance of the activity in this cell population can have severe damaging effects on the growth plate.

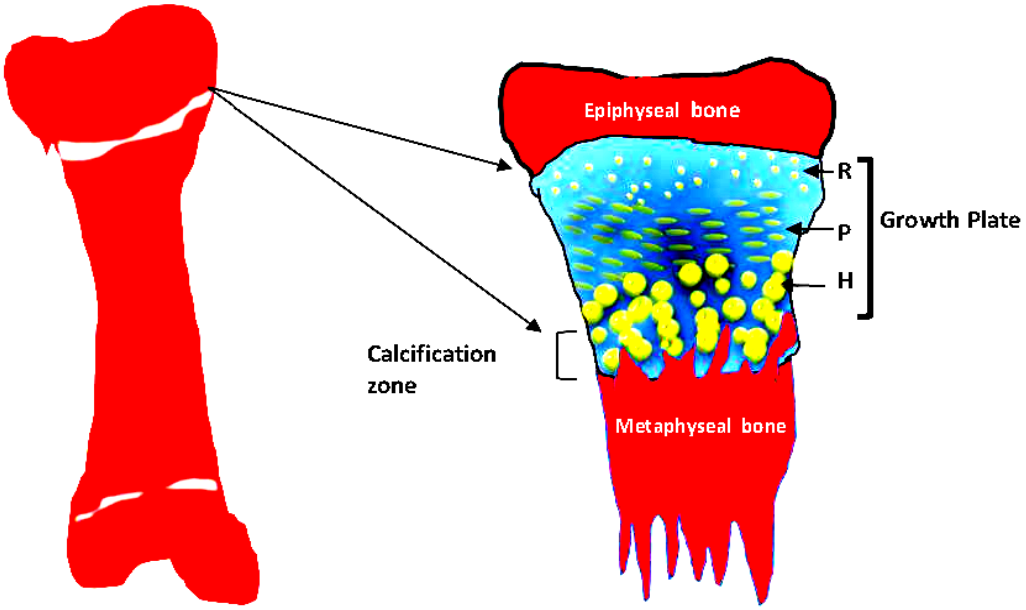

Figure 1. Growth plate cartilage in bone, showing three different zones of chondrocytes: (R) resting, (P) proliferative and (H) hypertrophic.

Proliferative chondrocytes, which are clearly larger than resting zone chondrocytes, display a flattened/discoid morphology and make columns parallel to the long axis of the bone. These cells highly and actively produce extracellular matrix (ECM) containing type-II and type-XI collagens (Hunziker and Schenk 1989) (Nilsson and Baron 2004), which maintain the integrity, function and shape of the growth plate. When proliferative chondrocytes lose their capacity to proliferate, they differentiate and enter the "hypertrophic phase" (Kember and Walker 1971) (Kember 1978). It is known that a broiler chicken chondrocyte requires approximately 21 hr to move from the proliferative phase to a terminally hypertrophic/differentiated phenotype (Thorp 1988), whereas the mean cycle time of proliferative chondrocytes in human and rat growth plates is approximately 20 and 2 days, respectively (Kember and Sissons 1976). As proliferative chondrocytes enter into hypertrophy, they further increase their size to a maximum level, increasing their intracellular volume 10-fold (Hunziker, Schenk et al. 1987).

Hypertrophic chondrocytes secrete large amounts of matrix proteins, vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) (Baron, Klein et al. 1994) (Gerber, Vu et al. 1999) (Haeusler, Walter et al. 2005). Interestingly, VEGF-A is only expressed in the hypertrophic zone in growth plate

cartilage and is key for inducing vascular invasion of blood vessels and bone cells into the hypertrophic cartilage from the metaphyseal side where cartilage is replaced with bone (Gerber, Vu et al. 1999) (Carlevaro, Cermelli et al. 2000) (Baron, Klein et al. 1994). Ultimately, hypertrophic chondrocytes undergo apoptosis (Farnum and Wilsman 1987) (Hatori, Klatte et al. 1995) or aberrant cell death (Erenpreisa and Roach 1998) (Roach and Clarke 2000).

In summary, most studies suggest that hypertrophic chondrocytes are removed via cell death from the growth plate, though some studies have suggested that chondrocytes trans-differentiate into osteoblasts. While apoptosis/aberrant cell death appears to be a part of the normal bone elongation process, any disruption of chondrocyte activity (either due to a disruption of normal cell division or excessive cell death) may lead to defective longitudinal bone growth.

2.3 CELL DEATH SIGNALING

Extensive studies characterizing cell death have increased our knowledge about various cell death signaling events. All multicellular organisms require apoptosis, the controlled death of cells, to regulate cell number in tissues and to eliminate individual cells that are no longer needed. During apoptosis, cells undergo nuclear and cytoplasmic condensation, and the plasma membrane blebs, i.e., breaks apart into membrane-enclosed particles (referred as apoptotic bodies) containing intact organelles and portions of the nucleus. Finally, these apoptotic bodies are rapidly recognized, ingested and degraded by phagocytes or neighboring cells. According to the Nomenclature Committee on Cell Death (NCCD), there are two types of mammalian cell death: apoptosis and necrosis. In addition, autophagy is considered a third mode of cell death.

It is well know that in mammalian cells, two major apoptotic signaling pathways exist: the intrinsic pathway, which is dependent on the mitochondria; and the extrinsic pathway, which is regulated through death receptors (DRs) that are present on the cell surface (**Figure 2)** (Kroemer 2003) (Choi and Benveniste 2004). A common downstream event such as caspase-8 of both extrinsic and intrinsic pathway activation may lead to caspase-dependent or caspase-independent signaling.

2.3.1 Extrinsic Apoptotic Pathway

As the immune system detects any abnormal markers on cell, it releases specific ligands, such as Fas and TNF, that bind and activate DRs located on the cell surface. This is followed by the formation of the death-inducible signaling complex (DISC), which results in the activation of pro-caspase-8. Activation of caspase-8 leads to activation of pro-caspase-3, which then cleaves target proteins and causes apoptosis (Chicheportiche, Bourdon et al. 1997).

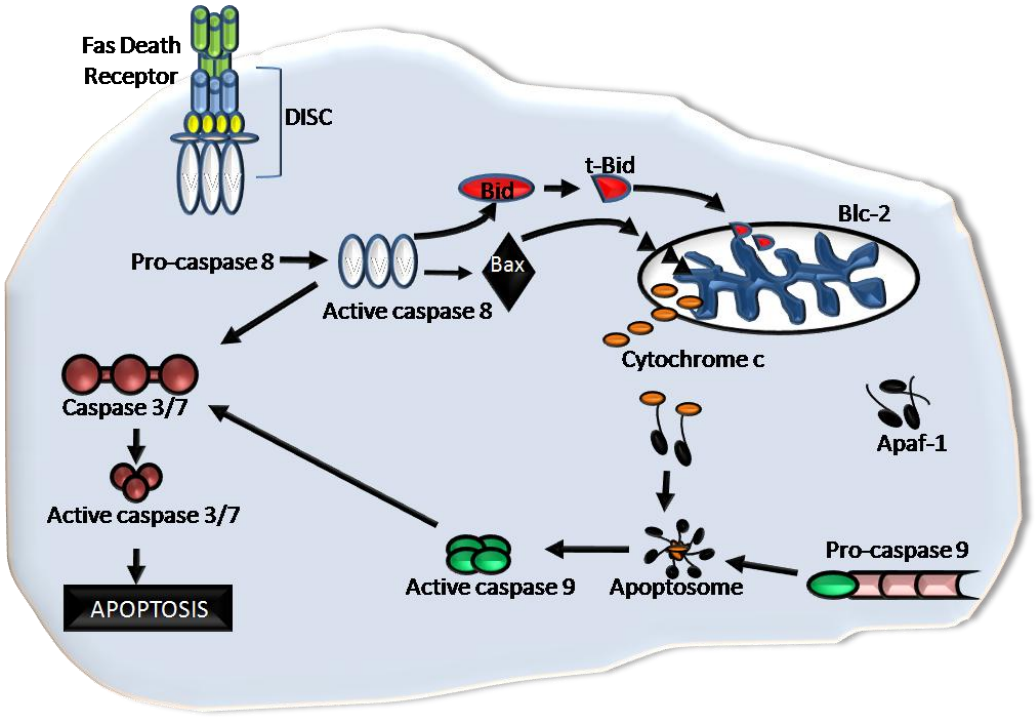

Figure 2. Extrinsic/intrinsic apoptotic signaling pathways and their interactions. Extrinsic cell apoptotic signaling is initiated through cell death receptors located in the cell membrane. Ligation of death receptors, such as Fas, is followed by the formation of the death-inducing signaling complex (DISC), which results in the activation of pro-caspase-8. The active caspase-8 can then directly activate caspase-3 to induce apoptosis or cleave Bid or Bax. Cleaved Bax or Bid translocates into the mitochondria, resulting in activation of mitochondrial apoptosis, where dysfunction of mitochondria is key for the execution of this type of apoptosis. After dysfunction of the mitochondria, the apoptosis can be caspase-dependent (via caspase-9 activation) or -independent (regulated via AIF), leading to DNA fragmentation.

2.3.2 Intrinsic Apoptotic Pathway

The intrinsic apoptotic pathway is triggered by direct damage to the cell from a wide range of factors, such as cellular stress, radiation, cytotoxic drugs and lack of essential growth factors, which may cause release of apoptogenic proteins from the mitochondria (Acehan, Jiang et al. 2002). Mitochondrial damage triggers cytochrome c release, leading to the formation of the apoptosome complex, which includes cytochrome c, Apaf-1 and pro-caspase-9 **(Figure 2)**. Activation of caspase-9 further leads to the activation of caspase-3, -6 and -7, resulting in cell death. Additionally, the balance between pro- and anti-apoptotic proteins also facilitates intrinsic apoptotic signaling. The activation of caspase-9 and its downstream events are controlled by Bcl-2 family proteins, which maintain the permeabilization of the outer mitochondrial

membrane (Danial and Korsmeyer 2004). According to the rheostat model of apoptosis regulation, the relative amount of pro-apoptotic versus pro-survival members of the Bcl-2 family is a critical determinant of the intrinsic cell death pathway (Wyllie 2010).

2.3.3 Autophagy

Autophagy was initially described as a fundamental survival strategy of cells and is an evolutionarily conserved mechanism by which long-lived proteins and damaged organelles are digested in lysosomes. Currently, however, in parallel with the apoptotic and necrotic forms of cell death, autophagy is considered a third form of cell death in which chromatin condensation is absent but is characterized by massive autophagic vacuolization of the cytoplasm (Klionsky and Emr 2000). All of these modes of cell death can be triggered by environmental contaminants, chemotherapeutic/toxic drugs and engineered nanomaterials, but the method of cell death is heavily dependent on cell type and exposure dose (Orrenius and Zhivotovsky 2006).

Recent reports indicate that despite cells presenting characteristics of autophagic cell death, they can still recover upon withdrawal of the death-inducing stimulus. Interestingly, it was recently reported that autophagy enhances cancer cell survival under conditions of stress/starvation and hence can function as a defense mechanism against various chemotherapeutic drugs (Abedin, Wang et al. 2007).

2.4 CHEMOTHERAPY

The modern concept of chemotherapy began in the 1940s with the use of nitrogen mustards and antifolate drugs (Chabner and Roberts 2005). However, mustard gas had already been used as a chemical warfare agent in World War I, and it was further studied during World War II. In a military operation which was carried out during World War II, a group of people were accidentally exposed to mustard gas, which caused a substantial decrease in their white blood cell count, as revealed in their medical examinations. This observation triggered a new concept of killing cancer cells, because mustard gas killed rapidly growing white blood cells. In 1942, Louis Goodman and Alfred Gildman used nitrogen mustard to treat a patient suffering from non-Hodgkin's lymphoma (Goodman, 1984). In 1955, the National Cancer Institute (USA) started the National Cancer Program, which is regarded as the first systematic program for drug screening (Chabner and Roberts 2005).

Currently, > 100 drugs are used as chemotherapeutics, either alone or in combination. These drugs vary widely in their chemical composition and the way they are used in the treatment of different diseases. The main goal of chemotherapy is always to cure a disease, but it can also be used to control the disease if a cure is not possible. Finally, if control is not possible, then the use of chemotherapy is intended for palliation (i.e., improving the quality of life of a patient).

2.4.1 Chemotherapeutic Drugs, Types and Effects on Bone Growth

Chemotherapy drugs can easily be divided into several groups based on their mechanism(s) of actions, chemical structure, and their interactions with other drugs. Briefly, the main classes of these drugs include *alkylating agents* (capable of inducing direct DNA damage) (Schwartz 1989), *anti-metabolites* (interfere with DNA and RNA production) (Zoli, Ulivi et al. 2005), a*nti-tumorantibiotics* (interfere with enzymes involved in DNA replication) (Dimarco, Gaetani et al. 1964) (Zaremba, Thomas et al. 2010), *topoisomerase inhibitors* (block topoisomerases) (Ishii, Katase et al. 1982) (Chen, Yang et al. 1984) (Ross, Rowe et al. 1984), *mitotic inhibitors* (known to interfere with the normal progression of mitosis) (Wibe, Oftebro et al. 1978)

(Musende, Eberding et al. 2010) and *corticosteroids/GCs* (Schulman 1950) (Hench, Kendall et al. 1950). The discovery of a protein degradation system via the proteasome, awarded the Nobel Prize in Chemistry in 2005 (Ciehanover, Hod et al. 1978), and the subsequent combined efforts of several laboratories resulted in the development of a new class of drugs called PIs (Schow and Joly 1997) (e.g., bortezomib (Velcade®)) (Hideshima, Richardson et al. 2001). Because PIs act differently and do not fit well into any of the other categories of chemotherapeutic drugs, we list them as *miscellaneous chemotherapy drugs.*

While chemotherapy is currently used to treat various diseases in children with a very high success rate, the long-term side effects of chemotherapy are also becoming obvious: it alters longitudinal bone growth, causes osteoporosis and frequent bone fractures that persist into adulthood (Schriock, Schell et al. 1991) (Halton, Atkinson et al. 1996). Thus, the question arises regarding whether the reported growth suppressive effects of chemotherapy are due to a direct interaction between drug(s) and bone tissue or via a systemic imbalance of hormones essential for normal bone growth, such as growth hormone/insulin-like growth factor-I (GH/IGF-I). Previous studies show that drugs such as corticosteroids influence the hypothalamic pituitary axis, causing systemic imbalance of hormones (which results into altered bone growth) (Allen 2002), and directly impair bone growth (Baron, Huang et al. 1992). We and others have shown that some chemotherapeutics directly target bone tissue without affecting the hypothalamic–pituitary axis because children treated with chemotherapy alone show no disturbances in growth hormone (GH) secretion (Samuelsson, Marky et al. 1997), and drugs such as PIs, the topoisomerase inhibitor etoposide, the anti-metabolite 5-fluorourocil (5-FU) and the alkylating agent cyclophosphamide can impair bone growth mechanisms directly (Xian, Cool et al. 2006) (Xian, Cool et al. 2007) (Zaman, Fadeel et al. 2008), (Zaman, Menendez-Benito et al. 2007).

2.5 IMPORTANCE OF GCs IN CHILDREN AND THEIR EFFECTS ON BONE GROWTH

GCs are vital steroid hormones (naturally-produced, or synthetically prepared) known to bind the GC receptor (GR) in mammalian cells. Cortisone was first given to a young woman suffering from rheumatoid arthritis (RA). Interestingly, the outcome of cortisone treatment in this patient and 15 other patients was very promising, and Hench reported these findings in 1949 while working at the Mayo Clinic in Rochester, Minnesota (Hench, Kendall et al. 1949). As these data were published, systemic administration of corticosteroids came into practice to treat various diseases, including asthma and rheumatic diseases. Surprisingly, all of these treatments resulted in similar positive effects, and in 1950, the Nobel Assembly awarded the Nobel Prize in Physiology or Medicine to Phillip Hench, Edward Kendall, and Tadeus Reichstein "for their discoveries relating to the hormones of the adrenal cortex, their structure, and biological effects".

GCs are widely used to treat inflammatory and autoimmune conditions, including a variety of life-threatening and disabling disorders. In the UK alone, > 250,000 people currently take systemic steroids for various conditions, and ≥ 10% of all children require some form of GC treatment during childhood (Mushtaq and Ahmed 2002). High doses of GCs are administered systemically and/or locally to children under various conditions, including RA, asthma, Crohn's disease and ulcerative colitis, and these children are at risk of severe GC side effects (Silva, Kater et al. 1997). Doses of dexamethasone, a widely used GC, range from 0.75 to 9 mg/day depending on the disease being treated and the response of the patient.

GCs are also widely used to treat all pediatric cancers, e.g., patients with acute lymphoblastic leukemia (ALL) (Mitchell, Richards et al. 2005) and Morbus Hodgkin (MH) (Felder-Puig, Scherzer et al. 2007). In 1971, Baxter et al. showed for the first time that GCs effectively kill lymphoma cells that express GR, and since that time, GCs have been widely tested/used in the treatment of a variety of cancers (Baxter, Harris et al. 1971).

2.5.1 Side Effects of GC Treatment

Despite the high degree of therapeutic efficacy of GCs, their use is associated with various side effects. These potential side effects include increased appetite, altered fat distribution (Cushing-like), obesity, headache, mood swings, hypertension, gastritis, diabetes mellitus, myopathy, osteopenia, hepatomegaly, immune suppression with a resultant increase in infections, and osteoporosis and bone growth impairment (Deshmukh 2007). Yeh et al. (2004) suggested not using early postnatal dexamethasone therapy for the routine prevention or treatment of chronic lung disease because it was found to be associated with substantial adverse effects on neuromotor and cognitive function in school-aged children (Yeh, Lin et al. 2004).

2.5.2 GC-induced Longitudinal Bone Growth Impairment

Both short-term and long-term use of GCs has been reported to retard the growth of bones, and familial deficiency of GCs has been reported to induce tall stature (Elias, Huebner et al. 2000). Bone formation is also altered by GCs, which is mainly due to depletion of mature osteoblasts caused by increased apoptosis and a decrease in proliferation/differentiation (Weinstein, Jilka et al. 1998). These observations indicate that excessive use or a deficiency of GCs can affect the bone. Further, GCs affect bone in a dose-dependent manner, and there is no dose that is considered completely safe (Da Silva, Jacobs et al. 2006).

The growth suppressive effects of short-term GC use are temporary, and growth is recovered soon after termination of GC treatment. However, the long-term use of GCs often results in severe irreversible side effects on bone, causing growth retardation (Magiakou, Mastorakos et al. 1994) (Yeh, Lin et al. 2004). The growth suppressive effects induced by GCs may vary from patient to patient, due to the amount of drug given to the patient, duration of treatment and the condition of the patient receiving treatment. The observation that exogenously administered GCs may impair longitudinal bone growth (Altman, Hochberg et al. 1992) (Allen 1996; Allen 2002)

(Smink, Koedam et al. 2002) led researchers to investigate the underlying mechanism behind this phenomenon.

Currently available data suggest that the growth-suppressing effects of GCs are multi-factorial and can be caused through both systemic regulation and direct effects on growth plate cartilage. Systemic bone growth retardation via GCs altering the GH/IGF-1 axis (Altman, Hochberg et al. 1992) (Allen 2002), as well as direct GC effects on the growth plate (Baron, Huang et al. 1992), highlight two distinct mechanisms of GC action. The fact that the GR is widely expressed in all tissues and in the growth plate cartilage (Silvestrini, Mocetti et al. 1999) (Abu, Horner et al. 2000) suggests that the direct and indirect bone growth retardation mechanisms of GC action may co-exist. Interestingly, a recent study has reported that GC treatment up-regulates GR in the growth plate cartilage, which results in decreased longitudinal bone growth in rats (Zhang, Wang et al. 2007). Similarly, several *in vitro* and *in vivo* studies have shown that GC treatment decreases the width of the growth plate due to decreased proliferative capacity of growth plate chondrocytes, increased chondrocyte differentiation and altered matrix production (Dearden, Mosier et al. 1986) (Mushtaq, Farquharson et al. 2002) (Annefeld 1992) (Silbermann and Maor 1978). Investigating any possible role of aberrant cell death or apoptosis, Silvestrini and co-workers reported that growth plate chondrocytes in rats are susceptible to apoptosis after long-term treatment with corticosterone (Silvestrini, Ballanti et al. 2000). In 2003, Chrysis et al. also reported an association between GC-induced apoptosis of growth plate chondrocytes and bone growth retardation (Chrysis, Ritzen et al. 2003). The decrease in the number of chondrocytes in the growth plate also alters ECM and collagen type-II, and this decrease in collagen type-II can further potentiate undesired apoptosis. Indeed, it has been shown that cartilage ECM lacking collagen II cannot support the survival of chondrocytes (Yang, Li et al. 1997).

In summary, the premature loss of resting/proliferative/hypertrophic chondrocytes by apoptosis or aberrant cell death may contribute to the incomplete resumption of growth often seen after prolonged GC treatment. This premature loss of chondrocytes may also be associated with further long-lasting effects on bone, such as a decreased number of total cells, less matrix production, and thereby, increased susceptibility to apoptosis.

2.5.3 Cell Death in Growth Plate Chondrocytes

The currently available data provide sufficient evidence of presence and regulation of apoptosis/cell death in the growth plate cartilage under normal bone elongation processes (Aizawa, Kokubun et al. 1997) (Bronckers, Goei et al. 1996) (Hatori, Klatte et al. 1995) (Chrysis, Nilsson et al. 2002). Although apoptotic cells have been detected in all three zones of growth plate cartilage (i.e., the resting, proliferative and hypertrophic zones), it is widely believed that only hypertrophic chondrocytes undergo frequent apoptosis. In 1997, Amling et al., Wang et al. and Krejewska et al. reported that caspase-3, anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax are expressed in the growth plate cartilage (Krajewska, Wang et al. 1997) (Amling, Neff et al. 1997) (Yang, Gu et al. 1997). Interestingly, Bcl-2 expression was found to be high in proliferative chondrocytes but very low in hypertrophic chondrocytes (Wang, Toury et al. 1997). The reported imbalance of Bcl-2 protein expression within the different zones of the growth plate cartilage indicates that some cells (e.g., proliferative chondrocytes) require anti-apoptotic factors to survive. However, when these proliferative chondrocytes become hypertrophic, the expression level of Bcl-2 is also decreased.

The importance of Bcl-2 in growth plate cartilage is also evident from studies reporting that mice lacking Bcl-2 exhibit premature chondrocyte maturation and differentiation, decreased growth plate thickness, short limbs and decreased total body length (Amling, Neff et al. 1997). In both PTHrP knockout mice and mice containing an activating mutation in FGFR3 (both of which result in chondroplastic conditions), it was noted that apoptosis increased in the growth plate chondrocytes (Amizuka, Henderson et al. 1996) (Legeai-Mallet, Benoist-Lasselin et al. 1998). Chrysis and co-workers further characterized the apoptosis in the growth plate cartilage of young rats (Chrysis, Nilsson et al. 2002) and concluded that apoptosis is developmentally regulated in normal growth plate cartilage, as they detected by caspase-3, caspase-6, Bcl-2, Bcl-x, p53, and Bax in different developmental stages. They also reported that in older animals with decreased growth rate and growth plate height, apoptosis is increased in hypertrophic chondrocytes. In summary, all of these reports indicate that

growth plate chondrocytes experience proper cell death signaling, which can be activated under different conditions.

2.5.4 GC-induced Cell Death in Growth Plate Chondrocytes

It is well known that GCs can induce cell death/inhibit proliferation in many cell types, such as in monocytes (Schmidt, Pauels et al. 1999), thymocytes (Marchetti, Di Marco et al. 2003), osteocytes, osteoblasts (Plotkin, Weinstein et al. 1999), articular chondrocytes (Nakazawa, Matsuno et al. 2002), skeletal muscle cells (Lee, Wee et al. 2005) and lymphoblastic leukemia cells (Bansal, Houle et al. 1989) (Laane, Panaretakis et al. 2007). However, it is also evident that GCs can render cancer cells more resistant to drug-induced apoptosis, such as in ovarian cancer cells and lung carcinoma (Chen, Wang et al. 2010) (Herr, Ucur et al. 2003). This observed GC-induced resistance to apoptosis in cancer may be partially due to inhibition of key molecules of the death receptor and the mitochondrial apoptosis pathway (e.g., caspase-9 activity and pro-apoptotic BID), resulting in blockage of caspase activity (Herr, Ucur et al. 2003).

Despite extensive work on the mechanisms GC action in the various cell types mentioned above, the mechanisms of GC-induced apoptosis in growth plate chondrocytes are not fully understood. Dexamethasone treatment of germ cells increases FasL expression and apoptosis, indicating that GCs can stimulate the CD95 death receptor pathway (Khorsandi, Hashemitabar et al. 2008), but in growth plate chondrocytes, it is still unclear if GCs directly activate the CD95 signaling pathway. The first evidence of apoptosis in growth plate chondrocytes after GC treatment was reported by the detection of TUNEL-positive cells, increased Bax and decreased levels of Bcl-2 expression (Sanchez and He 2002) (Mushtaq, Farquharson et al. 2002) (Chrysis, Ritzen et al. 2003). However, we performed the first detailed characterization of GC-induced apoptosis in proliferative chondrocytes and showed that apoptosis in these cells is mainly regulated through activation of the caspase cascade, which includes caspase-8 and -9 and suppression of the Akt-phosphorylation signaling pathway (Chrysis, Zaman et al. 2005).

2.5.5 Autophagy in Growth Plate Chondrocytes

Autophagy (i.e., type II programmed cell death) has been reported in growth plate chondrocytes, but its exact role is unclear. Erenpreisa and Roach (1998) and Roach and Erenpreisa (1996) reported that hypertrophic chondrocytes exhibit unusual, ultramicroscopic features. Although they observed condensed chromatin suggestive of apoptosis, the cellular morphology did not match that of cells undergoing apoptosis or necrosis. These authors noted an increase in the size of the endoplasmic reticulum and Golgi apparatus in terminal hypertrophic chondrocytes (Roach and Erenpreisa 1996) (Erenpreisa and Roach 1998). However, Srinivas and Shapiro (2006) noted that the these hypertrophic cells exhibit autophagic characteristics (Meijer and Codogno 2004) because they contain double-membrane vacuoles and display a loss of membrane structure and destruction of organelles. The ultramicroscopic characteristics of hypertrophic chondrocytes also resemble cells undergoing autophagy (Srinivas and Shapiro 2006). Watanabe, Bohensky et al. (2008) also reported that uncoupling proteins (UCPs) are expressed in the growth plate cartilage, with the highest expression in the hypertrophic zone, and suppression of UCP3 enhances the autophagy phenotype in chondrocytes. In summary, research on the regulation of autophagy in late growth chondrocytes demonstrates that genes responsible for triggering autophagy exist in chondrocytes, and this form of cell death can be induced within the growth plate cartilage under various conditions.

Interestingly, GC treatment has also been reported to induce autophagy in lymphoid leukemia cells via cytotoxicity (Grander, Kharaziha et al. 2009) (Laane, Tamm et al. 2009). In this context, we cannot rule out the possible existence of GC-induced autophagy in the growth plate chondrocytes. Because *in vivo* treatment with GCs dramatically decreases the size of hypertrophic chondrocytes and level of apoptosis is not dramatically high (suggesting that other forms of cell death may exist in these cells), it will therefore be interesting to further examine the markers of autophagy.

2.5.6 GC-induced Intrinsic Apoptosis in Growth Plate Chondrocytes

In 1998, Scaffidi et al. reported the existence of two distinct cell types that utilize discrete CD95 signaling pathways. According to their model, type-I cells undergo CD95-mediated apoptosis (caspase-8 activation via CD95) independent of mitochondria, whereas type-II cells require release of cytochrome *c* from the mitochondria (intrinsic pathway) to execute apoptosis (Scaffidi, Fulda et al. 1998).

Akt phosphorylation promotes cell survival and opposes apoptosis (Kennedy, Wagner et al. 1997) by a variety of routes. For example, the pro-apoptotic Bax protein is regulated by phosphorylation in an Akt-dependent manner, and this phosphorylation event blocks the effects of Bax on mitochondria by restricting Bax to the cytoplasm (Gardai, Hildeman et al. 2004). Similarly, pro-apoptotic Bid expression has also been reported to decrease upon Akt activation (Goncharenko-Khaider, Lane et al. 2010). The ability of the Akt signaling pathway to positively regulate chondrocyte maturation, proliferation, differentiation, cartilage matrix production, and cell growth in skeletal development (Rokutanda, Fujita et al. 2009) (Ulici, Hoenselaar et al. 2008) indicates its multiple modes of action and importance in chondrocytes. The cross-talk between Akt and Bcl-2 family proteins, such as Bax and Bid, suggests that apoptosis due to decreased phosphorylation of Akt can trigger mitochondrial mediated apoptosis/intrinsic apoptosis. The existing data on GC-induced apoptosis of growth plate chondrocytes shows that both caspase-8 and -9 are activated (Chrysis, Zaman et al. 2005). Although caspase-8 is often thought to be activated exclusively through the death receptor pathway, activation of caspase-8 can also occur in a mitochondria-dependent manner that is independent of the Fas-associated death domain (FADD) (Tsao, Su et al. 2008) (Wesselborg, Engels et al. 1999).

Furthermore, we have also shown that GC treatment *in vivo* in rats increases the levels of pro-apoptotic Bax and decreases the expression of Bcl-2 in growth plate cartilage (Chrysis, Ritzen et al. 2003); both of these proteins act at the mitochondrial level to trigger or block apoptosis. Further, tissue specimens from RA patients have higher levels of Bax than healthy controls (Hilbers, Hansen et al. 2003). In addition, strong Bax staining has also been observed in chondrocytes at sites of cartilage degradation (Hilbers, Hansen et al. 2003). The fact that the detected GC-

induced apoptosis in growth plate chondrocytes is not dramatically high (i.e., a reason underlying the poor detection of apoptosis) may be limitations of the methodology being applied. In most reports, TUNEL assays are widely used to detect apoptosis. However, as discussed earlier, it is possible that chondrocytes in the growth plate experience different modes of cell death (i.e., some cells are in the initial phase of apoptosis and do not display the terminal hallmarks such as DNA fragmentation when the assay is performed). It is also possible that some cells die due to autophagy. Furthermore, chondrocytes in the resting, proliferative and hypertrophic zones may display different levels of sensitivity to GCs and die prematurely, leaving no signs of apoptosis but decrease the number of cells in the entire growth plate.

In summary, the currently available data on growth plate chondrocytes suggest that these cells behave as type-II cells upon treatment with GCs, and disruption of apoptotic signals upstream of mitochondria before GCs target mitochondria may be one way to protect the growth plate chondrocytes from such undesired effects.

2.5.7 Prevention of GC-induced Growth Retardation

The identification and exploitation of new targets to inhibit apoptosis under different pathological conditions that feature excessive apoptosis (e.g., ischemia, arthritis and neurodegenerative diseases) remains a considerable focus of attention. Potential mechanisms of anti-apoptotic therapy may include stimulation of the inhibitors of apoptosis proteins, inhibition of the caspase cascade, poly [ADP-ribose] polymerase inhibition, stimulation of the PKB/Akt (protein kinase B) signaling pathway, and inhibition of Bcl-2 proteins such as Bax (Deveraux and Reed 1999) (Gagarina, Carlberg et al. 2008) (Mocanu, Baxter et al. 2000) (Zhou, Swanson et al. 2006) (Fujio, Nguyen et al. 2000) (Hochhauser, Kivity et al. 2003).

In chondrocytes, systemic or locally produced IGF-I plays a key role as a survival factor. Because GC treatment may alter both the local and systemic IGF-I levels/expression, strategies to prevent the effects of GCs on IGF-I expression (either systemically or locally) may have beneficial outcomes. Interestingly, a recent study shows that despite the absence of tissue IGF-I expression, maintaining long-term

elevation of the serum IGF-1 level can maintain the body size in its normal shape, skeletal architecture and mechanical function (Elis, Courtland et al. 2010). Several studies have also reported that GC treatment decreases the width of the growth plate. These GC growth plate damaging effects may be associated with decreased proliferation, increased apoptosis of growth plate chondrocytes and altered levels of GH (Ohyama, Sato et al. 1996) (Wehrenberg, Baird et al. 1989) and IGF-I, both systemically (Altman, Hochberg et al. 1992) (Allen 2002) and locally in the growth plate cartilage (Smink, Gresnigt et al. 2003).

The observations that patients with Laron syndrome (IGF-I deficiency) exhibit growth retardation and osteoporosis (Laron, Klinger et al. 1999) and that IGF-I and IGF-IR knockout mice display severe growth retardation (Baker, Liu et al. 1993) (Liu, Baker et al. 1993) indicate the importance of IGF-I in the regulation of longitudinal bone growth. Decreased circulating levels of IGF-I (systemically or locally) in the growth plate cartilage due to high doses of GCs may further sensitize chondrocytes to GC-induced apoptosis, which may alter longitudinal bone growth **(Figure 3)**.

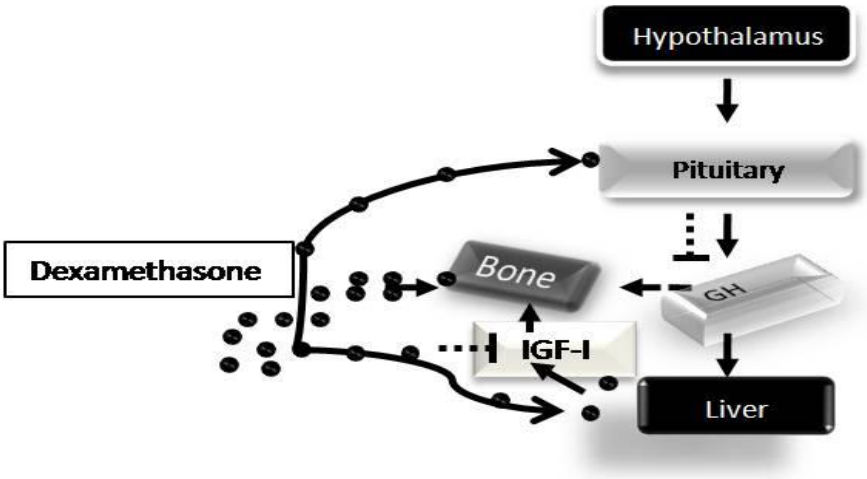

Figure 3. Schematic showing how GCs such as dexamethasone can alter IGF-I levels by directly acting on liver and via altering GH levels. In addition to altered levels of GH and IGF-1, dexamethasone can also directly act on growth plate cartilage in bone and thereby exert its growth inhibiting effects. After the systemic and local levels of GH and IGF-I are altered, the chondrocytes are more susceptible to any toxic effects of death stimuli such as dexamethasone.

We and others have previously reported that IGF-I can protect proliferative chondrocytes *in vitro* from dexamethasone-induced apoptosis (Macrae, Ahmed et al. 2007) by restoring the phosphorylation of Akt (Chrysis, Zaman et al. 2005), but it is still unknown if IGF-I can rescue organisms from GC-induced growth retardation. *In vitro* studies have shown that IGF-I stimulates DNA synthesis (Daughaday and Reeder 1966), sulfate proteoglycans, collagen production and proliferation (Ohlsson, Bengtsson et al. 1998). However, the IGF-I receptor also contributes to tumorigenesis, in part by promoting survival in tissue culture and *in vivo* (Resnicoff, Burgaud et al. 1995), which raises concerns for the frequent use of IGF-I in the treatment of a pathological condition/disease.

In an attempt to identify new drug(s) targeting GRs with anti-inflammatory effects, it has been reported that AL-438, a non-steroidal anti-inflammatory agent that acts through the GR, retains anti-inflammatory efficacy and has a reduced side effect profile on chondrocytes compared to other GCs (Owen, Miner et al. 2007). However, currently there are no studies investigating the effects of AL-438 on bone growth *in vivo.*

Therefore, it is extremely important to investigate and identify new molecular targets to promote the survival/proliferation of chondrocytes without altering the treatment used to combat a disease.

2.6 USE OF PIs IN CHILDREN AND THEIR EFFECTS ON BONE GROWTH

From the 1960s to the 1980s, the majority of researchers focused on examining nucleic acids and the translation of their encoded information, but little attention was given to protein degradation because it was regarded as a dead-end process. However, the discovery of the ubiquitin (Ub) pathway revolutionized this field because it answered many questions concerning the degradation of intracellular proteins. In 2004, to Avram Hershko, Aaron Ciechanover, and Irwin Rose were awarded the Nobel Prize in Chemistry for their discovery of Ub and the biochemistry of its conjugation to substrate proteins.

2.6.1 Development and Use of PIs in Children

A number of synthetic and natural PIs have been developed after it was found that proteasome inhibition can effectively kill cancer cells (Chandra, Niemer et al. 1998). Currently, there are five major classes of specific PIs: peptide aldehydes, peptide vinyl sulfones, peptide boronates, peptide epoxyketones and beta lactones. Recently, several new and promising compounds, such as salinosporamide A (formerly NPI-0052) and carfilzomib (PR-171) (Feling, Buchanan et al. 2003) (Kuhn, Chen et al. 2007), have been discovered and are under investigation for their potential use against various types of cancers. Indeed, early results from an international phase-III study in relapsed multiple myeloma patients presented in the "New England Journal of Medicine, June 16, 2005, in abstract" showed that bortezomib (also known as PS341 or Velcade^{TM}, developed by Millennium Pharmaceuticals and Johnson & Johnson Pharmaceutical Research & Development), a peptide boronate inhibitor of the 26S proteasome, is more effective than the conventional treatment of high-dose dexamethasone at delaying disease progression (Richardson, Sonneveld et al. 2005). To date, among all available PIs, only bortezomib has been approved by the Food and Drug Administration (USA) for third-line treatment of multiple myeloma patients due to its profound anti-tumor effect (Richardson, Sonneveld et al. 2005; Richardson, Mitsiades et al. 2006).

Because bortezomib was shown to have strong antitumor activity at nanomolar concentrations in a variety of cancerous cell lines in preclinical studies, it was approved for the treatment of myeloma patients (Richardson, Mitsiades et al. 2006). Thereafter, clinical trials were initiated in children (phase-I and phase-II in progress) to treat various childhood cancers (i.e., refractory leukemia, optic glioma, osteosarcoma, hepatoblastoma, neuroblastoma, adenocarcinoma, Wilms' tumor, and rhabdomyosarcoma) (Horton, Pati et al. 2007) (Blaney, Bernstein et al. 2004) (Messinger, Gaynon et al. 2010). Based on phase-I clinical trials, it was reported that bortezomib is well tolerated in children with recurrent or refractory solid tumors, and recommendations were made to use 1.2 mg/m^2/dose for phase-II trials, which are currently in progress. Furthermore, the efficacy of bortezomib as a single agent or in combination with other drugs is also being extensively studied in patients with multiple myeloma and other hematological malignancies. In one such example, it was recently reported that the combination of dexamethasone and bortezomib in patients with relapsed and/or refractory myeloma who had suboptimal responses to bortezomib alone is associated with improvement in treatment responses without prohibitive toxicity (Jagannath, Richardson et al. 2006).

2.6.2 *What is a Proteasome and How Does it Work?*

The proteasome is a large proteolytic complex that resides in the nucleus and cytosol of all eukaryotic cells and is actively involved in protein degradation. Ub is a small protein modifier that is covalently conjugated to proteins destined for proteasomal degradation because the proteasome preferentially binds to and degrades ubiquitinated proteins (Voges, Zwickl et al. 1999). Misfolded/aberrant proteins, which can be potentially dangerous for cells via the formation of insoluble aggregates, are also targeted for proteasomal degradation.

The Ub/proteasome system (UPS) is a complex and highly organized cascade of enzymatic reactions that select, mark, and degrade proteins in cells. To execute protein degradation, proteins are modified by Ub and thereby marked for degradation (Glickman and Ciechanover 2002). The conjugation of Ub to proteins helps them to be recognized by the 26S proteasome, a large proteolytic complex that degrades ubiquitinated proteins into small peptides. The binding of a chain of Ub to a

target protein requires three enzymatic components. These enzymes include E1 (which is a Ub-activating enzyme), E2s (which prepares Ub for conjugation) and E3, a Ub-protein ligase that is a key enzyme and helps to recognize a specific protein substrate and catalyzes the transfer of Ub to the protein for tagging **(Figure 4).** Another ubiquitination factor, E4 (which was discovered after E1-3) (Koegl, Hoppe et al. 1999), does not participate in the ubiquitin enzyme thioester cascade or interact with the substrate directly. However, in some cases, E4 is involved in elongating the poly-ubiquitin chain and thereby triggering protein degradation.

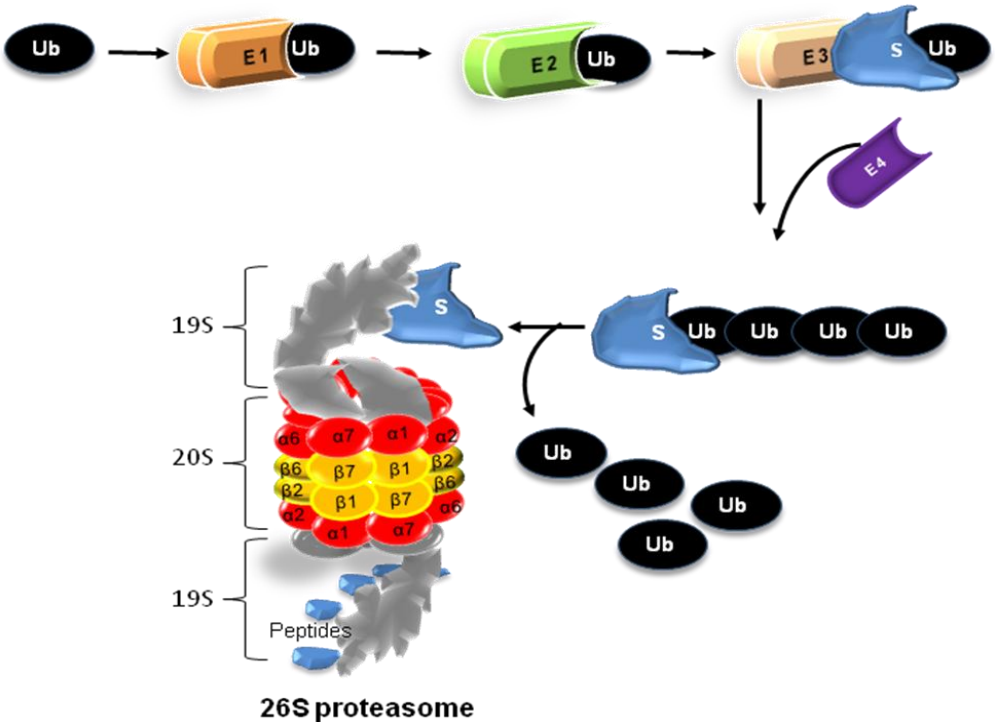

Figure. 4. Schematic diagram showing the structure of the 26S proteasome and the ubiquitin proteasome system involved in protein degradation. In the first step toward protein degradation, Ub is activated by E1, and this activated Ub is then transferred to E2. As the protein substrate (S) is recognized by E3, Ub is then transferred from E2 to the S. All of these events are repeated several times and result in the formation of a poly-Ub chain. Ub-tagged proteins are recognized by the 19S regulatory complex, where the Ub tags are removed. Although E4 does not always participate in the Ub cascade, it is involved in creating the poly-Ub chain in some cases.

The critical involvement of the UPS in the regulation of a number of cellular processes, as well as strict protein quality control, suggests that interference with this process may be harmful for cells. Indeed, *in vitro* studies conducted by Chandra et al. (1998)

confirmed the notion that cells normally undergo apoptosis when cultured in the presence of PIs, making these agents attractive candidates to kill cancer cells (i.e., a novel cancer therapy).

2.6.3 Proteasome Structure

The proteasome, a cylindrical structure of ~2 MDa in size, is composed of two complex components. The first component is the cylindrical 20S core particle (20S proteasome), and the second component is 19S cap particle, which is attached to the both ends of the 20S proteasome to yield the 26S proteasome (**Figure 4**). (Orlowski 1990) (Coux, Tanaka et al. 1996). After formation of a poly-Ub chain, the Ub-tagged proteins are transported to the proteasome and are quickly recognized by the 19S regulatory complex, where the Ubs are removed. Next, ATPases with chaperone-like activity at the base of the 19S regulatory complex unfold the protein substrates and push them into the 20S proteasome cylinder (Kloetzel 2001) (Braun, Glickman et al. 1999).

The 20S proteasome subunit (ring-shaped, 700 kDa) is composed of two α and two β rings. Each ring consists of seven α (21 kDa) and seven β (31 kDa) proteins (Tanaka 1998). After the protein substrate is unfolded, it enters the inner chamber without Ub, where it is hydrolyzed by active proteolytic sites located on six β-subunits and broken down into oligopeptides of 3-25 amino acids in length (Pickart and VanDemark 2000) (Groll and Huber 2003) (**Figure 4)**.

2.6.4 Side Effects Associated With PIs

The most prevalent side effects of PIs, thrombocytopenia and peripheral neuropathy, have been reported in treated patients (Blaney, Bernstein et al. 2004). Similarly, in a case report of a 79-year-old female patient with multiple myeloma and no prior history of cardiac disease, she developed an acute myocardial infarction on day 5 after receiving bortezomib and dexamethasone (Takamatsu, Yamashita et al. 2010). However, the cytotoxic effects of bortezomib on immune-competent cells have also been observed, suggesting a broad suppressive role for proteasome inhibition in the immune system (Wang, Ottosson et al. 2009). In a recent study it was reported that

bortezomib enhances susceptibility to viral infection (Basler, Lauer et al. 2009) and disrupts tumor necrosis (TNF)-related apoptosis-inducing ligand (TRAIL) expression, as well as TRAIL-dependent and/or -independent pathway-mediated killing of myeloma cells. This suggests that bortezomib may potentially hamper natural killer(NK)-dependent immunosurveillance against tumors in patients treated with this drug (Feng, Yan et al. 2010). In pre-clinical studies investigating the effects of bortezomib on bone growth, we also reported that linear bone growth is severely altered in young mice treated with bortezomib (Zaman, Fadeel et al. 2008). Thus, all of these studies suggest that bortezomib should be carefully monitored for any further potential side effects on other tissues.

2.6.5 PIs and Regulation of Apoptosis

The UPS plays an important role in the regulation of cellular proteins and degradation of proteins involved in cell cycle control, transcription, proliferation, differentiation, apoptosis, cell adhesion, angiogenesis and tumor growth. Preclinical studies show that proteasome inhibition exerts antitumor effects in a variety of cell lines, such as CNS malignancies, non–small cell lung cancer, leukemias, lymphomas, neuroblastoma, rhabdomyosarcoma, Ewing's sarcoma and colon, ovarian, renal, and prostate carcinomas (Omura, Matsuzaki et al. 1991) (Fenteany, Standaert et al. 1994) (Mugita, Honda et al. 1999) (Soldatenkov and Dritschilo 1997). In the first detailed characterization of the UPS, King et al. (1996) reported that key regulatory proteins such as NF-kß, p53 and the cyclin-dependent kinase inhibitor p21 are affected by inhibiting UPS (King, Deshaies et al. 1996). Since the development of PIs, new molecular targets in a variety of cells have been reported. For example, proteasome inhibition has been reported to induce reductions in the anti-apoptotic protein c-FLIP in Renca cells (murine renal cancer) (Sayers, Brooks et al. 2003), Bcl-2 phosphorylation in H460 cells (human lung carcinoma) (Ling, Liebes et al. 2002), synergistic effects on TRAIL and TNF-alpha in prostate cancer (An, Sun et al. 2003), and release of SMAC, AIF, and cytochrome c in cells obtained from patients with chronic lymphocytic leukemia (Pahler, Ruiz et al. 2003). In hepatocellular carcinoma cells, bortezomib-induced suppression of phospho-Akt leading to apoptosis has also been reported (Chen, Liu et al. 2010). Similarly, the influence of bortezomib on multi-drug-resistant human neuroblastoma cell lines characterized by P-glycoprotein expression and p53 mutation

has also been explored (Michaelis, Fichtner et al. 2006). Interestingly, even nanomolar concentrations of bortezomib inhibit vessel formation in neuroblastoma xenografts and the cell cycle, as well as induce apoptosis in chemosensitive and chemoresistant cells (Michaelis, Fichtner et al. 2006). In summary, these findings suggest that proteasome inhibition activates multiple cell death signaling pathways (extrinsic and intrinsic apoptosis) depending on cell type and dose.

2.6.6 Effects of PIs on Longitudinal Bone Growth

Late side effects are caused by the damage that chemotherapy exerts on normal/healthy cells in treated individuals. The main aim of chemotherapy is to target cancer cells that grow quickly, but in children, normal cells in some tissues such as bone also grow fast and are easily targeted as well. Indeed, decreased bone growth during childhood cancer treatment is a common problem, but most patients resume normal growth after treatment. For many years after the use of chemotherapy, regular follow-up exams are important for adults and children.

Because PIs are not ranked in the list of conventional chemotherapeutics due to their novel mechanisms of actions, it is not possible to speculate on their effects on longitudinal bone growth in children. Thus, investigation of such effects on bone growth in fast growing individuals is a key question that must be addressed.

To date, only two studies show a direct link between longitudinal bone growth and impairment of the Ub/proteasome system. In the first study, Wu and De Luca (2006) investigated the link between longitudinal bone growth and proteasome inhibition *in vitro* with cultures of fetal rat metatarsal bones. They found that bone length decreased after treatment with proteasome inhibitor-I (PS-I). They also showed that PS-I treatment *in vitro* increases the expression of beta-catenin (a negative regulator of chondrogenesis) and reduces the DNA binding of nuclear factor kappaB, a transcription factor that stimulates growth plate chondrogenesis (Wu and De Luca 2006). Further, we investigated the effects of the PIs MG262, lactacystine and bortezomib both *in vitro* and *in vivo* and found that proteasome inhibition induces severe and irreversible bone growth retardation (Zaman, Menendez-Benito et al. 2007) (Zaman, Fadeel et al. 2008). Surprisingly, young mice treated with the peptide boronate

PI MG262 remained growth retarded 45 days after termination of the treatment, and cultures of fetal rat metatarsal bones displayed permanent growth retardation (Zaman, Menendez-Benito et al. 2007).

Finally, these reports suggest that impairment of the UPS exerts severe side effects on the growth plate cartilage, causing irreversible growth retardation of bones. Furthermore, the inhibitory effects of various PIs (i.e., PS-1, lactacystine, MG262 and bortezomib) on bone growth demonstrate the significance of the UPS in regulating longitudinal bone growth.

2.6.7 PIs and Cell Death in Growth Plate Chondrocytes

To date, there are very few studies investigating if proteasome inhibition triggers apoptosis in chondrocytes. Kuhn and Lotz initially reported that chondrocytes from articular cartilage undergo apoptosis if challenged with the PI MG132. Proteasome inhibition enhanced CD95-dependent cell death by NF-kappaB inhibition at and/or downstream of caspase 8 activation without caspase 9 activation (Kuhn and Lotz 2001) and decreased collagen type-II (Yu, Kim et al. 2010). In contrast, it has also been reported that proteasome inhibition enhances intracellular expression of Hsp70 and protects chondrocytes from cellular injuries caused during osteoarthritis (Grossin, Etienne et al. 2004).

Investigating the effects of proteasome inhibition on growth plate chondrocytes (and thereby longitudinal bone growth), Wu and De Luc showed that PS-1 reduces longitudinal bone growth, which was associated with decreased chondrocyte proliferation and hypertrophy/differentiation, as well as increased levels of apoptosis, in chondrocytes (Wu and De Luca 2006). They also showed that PS-I treatment *in vitro* increases the expression of beta-catenin (a negative regulator of chondrogenesis) and reduces the DNA binding of nuclear factor kappaB, a transcription factor that stimulates growth plate chondrogenesis. Recently, we also showed that PI treatment *in vitro* and *in vivo* causes growth retardation. Furthermore, we observed that growth retardation is associated with high levels of apoptosis in resting zone chondrocytes. The detailed characterization of chondrocyte apoptosis revealed that apoptosis was both caspase-dependent and -independent. Both p53 and AIF were up-regulated upon

proteasome inhibition, and silencing p53 and AIF blocked apoptosis induced by proteasome inhibition (Zaman, Menendez-Benito et al. 2007). We further characterized the apoptotic signaling in growth plate chondrocytes and found activation of caspase-8, -9, and -3. Bortezomib treatment in growth plate chondrocytes also induced activation of the pro-apoptotic protein Bax (via conformational changes and its subsequent translocation into mitochondria) and decreased mitochondrial membrane potential (data not published paper-IV). In summary, the presently available limited data about effects of proteasome inhibition on growth plate chondrocytes indicate that these cells are highly sensitive to the UPS. More specifically, resting zone chondrocytes are very susceptible to apoptosis caused by impairment of the UPS, which results in severe growth retardation of bones.

2.6.8 Prevention of Growth Failure Caused by PIs

Our data on growth plate chondrocytes showed that proteasome inhibition activates p53, and silencing of this protein can protect chondrocytes from apoptosis. Further, a small molecule inhibitor of p53 has been shown to prevent the side-effects of cancer therapy in mice (Komarov, Komarova et al. 1999), suggesting that the simultaneous administration of a pro-apoptotic drug and a cytoprotective agent may be a feasible and advantageous chemotherapy approach. This treatment strategy is helpful in individuals where p53 is mutated and drugs given to the patient are known to kill cancer cells independent of p53. For instance, PIs can induce both p53 dependent and independent apoptosis. The combination of p53 inhibitors and PIs in cancer cells where p53 is non-functional due to mutation should not interfere with the PIs' ability to induce apoptosis. Additionally, normal cells will be protected, because apoptosis in these cells is p53-dependent. However, a comprehensive characterization of cell death signaling after proteasome inhibition will help us to identify new molecular targets for the prevention of undesired side effects.

3 AIMS OF THESIS

The main aim of this thesis is to characterize glucocorticoids such as dexamethasone and novel proteasome inhibitors for their effects on chondrocyte cell death signaling and longitudinal bone growth.

More specifically:

- To investigate Dexamethasone-induced apoptosis in proliferative chondrocytes **(PAPER-I)**.

- To identify new molecular targets for the prevention of bone growth retardation caused by Dexamethasone **(PAPER-II)**.

- To investigate the effects of proteasome inhibitors on longitudinal bone growth and apoptosis of growth plate chondrocytes **(PAPER-III)**.

- To characterize bortezomib-induced longitudinal bone growth retardation and apoptosis in growth plate chondrocytes (**PAPER-IV)**.

4 METHODS

The following methods were used and are briefly discussed in this section. Please refer to individual papers for more details of each method.

4.1 Experimental Models

- Cell cultures (Human and rat)
- Gene silencing *in vitro* by using siRNAs
- Organ cultures of fetal rat metatarsal bones
- Human growth plate biopsies
- Animal models (normal and genetically modified mice)

4.2 Experimental Methods

- PCR
- Cell proliferation/viability (BrdU and WST-1 assay)
- Caspase-3 assay
- Cell death detection ELISA and TUNEL assay
- Western blot/immunoprecipitation
- Immunohistochemistry/Immunocytochemistry
- Flowcytometry
- Digital automatic cell counting
- Measurement of longitudinal bone growth (organ cultures of fetal rat metatarsal bones *in vitro* and X-rays of femur/tibia *in vivo*)
- Serum IGF-I, Growth plate morphometry, van Gieson/Alcian blue staining**)**

4.1.1 Cell Cultures (PAPER-I, II, III, IV)

HCS-2/8 chondrocytic cell line (human)

HCS-2/8 cells, a chondrocytic cell line (Takigawa, Tajima et al. 1989) derived from a well-differentiated type of human chondrosarcoma, is well characterized as a cell culture model, and it is widely used to study chondrocyte proliferation/differentiation. The cell line expresses all the characteristics of proliferative chondrocytes, and after 3 weeks in culture, this cell line exhibits characteristics of hypertrophic/differentiated chondrocytes, such as expression of type-

X collagen and nodules formation. In paper-I, II and III, HCS-2/8 cell line was used a model for proliferative chondrocytes.

RCJ3.1C5.18 chondrogenic cell line (rat):

To validate our data obtained from HCS-2/8 chondrocytic cell line (human), we also have used another non-transformed/non-cancerous cell line named "RCJ 3.1C5.18". The RCJ 3.1C5.18 (C5.18) is a non-transformed clonal chondrogenic cell line (derived from fetal *rat calvaria*) is a mesenchymal stem cell system that does not require any biochemical or oncogenic transformation. These cells spontaneously and sequentially undergo chondrocyte differentiation and express resting, proliferative, and terminally differentiated chondrocyte phenotypes in a sequence that mimics that of growth plate chondrocytes (Grigoriadis, Heersche et al. 1996; Lunstrum, Keene et al. 1999). These cells sequentially acquire from 5-7 days of culture markers of chondrocytic differentiation (type II collagen/proteoglycans synthesis), and at 10 and 14 days of culture, these cells express markers of terminal differentiation (type-X collagen and alkaline phosphatase activity). Although this is an *in vitro model, the* morphology, histochemical markers and the temporal sequential acquisition of the chondrocytic phenotype in these cells appears to be identical to the chondrogenesis process that takes place *in vivo*, making these cells ideal and unique for studying chondrocyte cellular and molecular regulation at different stages of differentiation. In paper-III and IV, RCJ 3.1C5.18 cell line was used a model for resting/early differentiated, proliferative, and hypertrophic chondrocytes.

4.1.2 Gene Silencing in vitro by Using siRNA (Paper-II, III)

To test our hypothesis in paper-II and III that certain pro-apoptotic genes, such as Bax, Bid, p53 and AIF, play a key role in triggering apoptosis under different conditions in chondrocytes, we explored the possibility of using Small interfering RNA (siRNAs), a class of double-stranded RNA molecules, 20-25 nucleotides in length. siRNA interferes with the expression of a specific gene in the cells. Any gene of interest in which the sequence is known can be targeted based on complementary sequences with an appropriately tailored siRNA. We used

commercially pre-designed/tested siRNAs against p53 and AIF in our cell culture model system and were able to achieve >80% transfection efficiency (verified by western blotting) by incubating the cells with siRNAs for 24-48 hrs. Scrambled siRNA was used as negative controls in all experiments. For details, please refer to paper-II and III.

4.1.3 Organ Cultures of Fetal Rat Metatarsal Bones and Measurement of Longitudinal Bone Growth (Paper-II, III and IV)

To investigate the local/direct effects of Dexamethasone and proteasome inhibitors on bone growth, we used organ culture model system of fetal rat metatarsal bones (Paper-II, III and IV). It is a well-established model system of fetal rat metatarsal bones in which the direct effects of external stimuli can be observed under well-controlled conditions. For protocol details, please refer to paper-II, III and IV. The study was approved by the local ethics committee at Karolinska Institutet (Stockholm, Sweden).

The longitudinal bone growth was measured by using imageJ software (NIH) or MicroImage software. The pictures of bones in culture were taken and culture medium was changed on day 0, 2, 5, 7, 9 and 12. The day of dissection was identified as day 0 in culture. At the time of terminating experiment, the bones were labeled with BrdU (for details, please refer to paper-II, III and IV).

4.1.4 Human Growth Plate Biopsies (Paper-II, IV)

Most of our *in vitro* assays on the sensitivity of growth plate chondrocytes to apoptosis were obtained from rat chondrocytic cell line/fetal rat metatarsal bones or human chondrosarcoma cells. To check the response of human non-transformed growth plate chondrocytes to dexamethasone and bortezomib, we decided to use human growth plate cartilage biopsies. To use the human growth plate, tissue samples were obtained during epiphyseal surgery (to limit the bone growth in patients with leg length differences or predicted extreme tall stature). Informed consent and permission was acquired before collecting biopsies. The growth plate biopsies were collected by a bone marrow biopsy needle (Gallini Biomid, size 7G 10cm, Apgar, Denmark). Next, the biopsies were transferred to Falcon tubes (size, 50

ml) containing DMEM-high glucose and 20 µg/ml gentamicin and kept on ice. The biopsies were cut under sterile conditions into thin slices (~½-1 mm), and each biopsy was transferred into individual 24-well plates containing culture medium. The same culture medium was used in cultures of fetal rat metatarsal bones except for DMEM-high glucose and kept in +37ºC with 5% CO_2. After the incubation period of 24 hr with/without test drug(s), samples were fixed in 4% formaldehyde for 24 hr and decalcified in 10% EDTA, pH 7.8, for 24 hrs at 4 ºC. Samples were embedded in paraffin, cut into 4-µm-thick slices, and mounted on "super-frost glass" slides for further analysis.

4.1.5 Animal Models (Paper-II, III)

Bone growth studies in Bax deficient mice:

In paper-II, to test our hypothesis whether pro-apoptotic protein Bax is involved in dexamethasone-induced apoptosis of chondrocytes and bone growth retardation, we used Bax-deficient mice (male and female). Briefly, heterozygous C57BL/6 mice were obtained from Jackson Laboratories http://jaxmice.jax.org) and breeding was performed to obtain homozygous animals. Genotyping was done as described by the Jackson Laboratories, and homozygous (male and female), 30-32-day-old mice were used in the experiment. The animals received injection of dexamethasone (2 mg/Kg body weight, dissolved in 0.1 ml saline) or 0.1 ml saline subcutaneously in the neck for 28 days. The wild-type animals were also used in a similar manner as in Bax deficient mice. The protocol received approval of the local committee for animal ethics, Stockholm, Sweden. The animals were weighed, and the longitudinal bone length (left femur and tibia) was measured by X-ray at the beginning until the end of the experiment on weekly basis. All animals received 200 µl intraperitoneal injection of BrdU (10 mg/ml), 15 and 2 hrs before killing, and blood serum was also collected. The tibiae were carefully dissected and cleared from muscle and immediately fixed in 4% formaldehyde for 48 hr at 4 ºC. After decalcification in 10% EDTA, tibia from each animal was embedded in paraffin, and sections were made for apoptosis analysis.

Rat tibia growth plate (Paper-II):

We previously showed that dexamethasone induces growth retardation in rats (Chrysis, Ritzen et al. 2003), an effect associated with increased apoptosis of growth plate chondrocytes. Taking advantage of this study, we used the growth plate samples from these animals and stained them to detect if there was any activation of Bax with conformational changes, as Bax with conformational changes has been reported to induce mitochondrial injury leading to intrinsic apoptosis. These animals (7-week-old male Sprague Dawley rats, B&K Universal, Sollentuna, Sweden) were given dexamethasone (5 mg/kg body weight, s.c.) for 7 days, whereas the control group received vehicle only. On each slide, two sections were mounted, one from each group, so that all samples were treated under the same conditions during immunohistochemistry.

A transgenic mouse model of the ubiquitin/proteasome system (Paper-III):

We evaluated the *in vivo* effect of proteasome inhibitor MG262 using a reporter mouse model for the ubiquitin/proteasome system (Lindsten, Menendez-Benito et al. 2003) developed by Dr. Nico Dantuma's group (MTC, Karolinska Institutet). These mice express the ubiquitin^{G76V}-green fluorescent protein (Ub^{G76V}-GFP) that is constitutively targeted for ubiquitin-dependent proteasomal degradation by the presence of an ubiquitin fusion degradation signal. Tissues from these mice thus display low GFP fluorescence unless the cells fail to degrade the Ub^{G76V}-GFP proteasome substrate as a consequence of functional impairment of the ubiquitin/proteasome system. Low doses of the proteasome inhibitor, MG262, were administrated to 5-week-old male Ub^{G76V}-GFP/2 mice. The mice received intraperitoneal injections of 0.2 µmol MG262/kg body weight on days 1, 3 and 5 and were killed 24 hrs after the final injection. The femur and tibia were removed, fixed in 4% formaldehyde and embedded in paraffin. The sections (femur and tibia) were analyzed for GFP accumulation under fluorescent microscope.

4.2 EXPERIMENTAL METHODS

4.2.1 PCR, for Type-X Collagen and Genotyping

In paper-I, to rule out whether dexamethasone treatment triggers differentiation of proliferative chondrocytes in hypertrophic chondrocytes, we used Reverse-Transcriptional PCR (RT-PCR) to detect type-X collagen. Furthermore, in paper-II, this method was also used for genotyping of Bax heterozygous and homozygous animals, exactly as described in protocol provided by Jackson Laboratories on http://jaxmice.jax.org.

4.2.2 Cell Viability/Proliferation Assay (BrdU and WST-1 assay) (Paper-I,III, IV)

The measurement of cell viability and proliferation has become a key technology in drug(s) screening/discovery. In our studies, the effects of dexamethasone and proteasome inhibitors on chondrocytes viability/proliferation were assessed by two methods: a WST-1 cell viability/proliferation assay and a bromodeoxyuridine (BrdU) incorporation assay (Roche Diagnostic GmbH). BrdU ELISA is used for the semi-quantitative measurement of BrdU incorporation in newly synthesized DNA during DNA synthesis. In contrast, WST-1 is a ready-to-use substrate that measures the metabolic activity of viable cells and is suitable for measuring cell proliferation, cell viability or cytotoxicity. We used both methods to validate the effects on cell viability/proliferation if the data conflicted with the DNA fragmentation ELISA assay.

In paper-I and IV, we investigated the time- and dose-dependent effects of dexamethasone and bortezomib on chondrocyte viability/proliferation and therefore used the WST-1 assay. This assay is based on a water-soluble tetrazolium salt that is cleaved to formazan by mitochondrial enzymes and measured by ELISA. The amount of the formazan dye formed is directly correlated to the number of metabolically active cells. For more details, please refer to paper-I and IV.

In paper-III, we quantified cell proliferation by looking at DNA synthesis by means of BrdU incorporation by using ELISA kit. Briefly, HCS-2/8 chondrocytes in the proliferation stage were cultured in 96-well plates and treated with different

concentrations of proteasome inhibitors, such as lactacystine and MG262. After incubation with these drugs for a desired period, BrdU was added in the cell culture medium and further incubated for 3 hrs. After incubation, the cells were fixed and processed with further steps, and finally, color development was measured by using ELISA reader, according to the manufacturer's instructions (provided in the kit).

4.2.3 Caspase-3 Fluorometric Aassay (Paper-III)

To validate the proteasome inhibitor-induced apoptosis in chondrocytes, we determined active caspase-3 by using the fluorogenic peptide substrates, DEVD-AMC (aminomethylcoumarin; 50 µM, Biomol, Plymouth Meeting, PA). The cell lysates from chondrocytes and substrates were combined in a reaction buffer and real-time measurements of enzyme-catalyzed release of AMC were obtained using a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden) operating with Genesis software (Labsystems). Fluorescence was measured every 70 seconds during a 30-minute period.

4.2.4 Cell Death Detection ELISA, Cytochrome c ELISA, TUNEL assay and Digital Automatic Cell Counting (Paper I-IV)

In paper-I, II, III and IV, the detection and quantification of cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleasomes) were performed by a photometric enzyme-immunoassay (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics GmbH, Germany), and details are mentioned in paper-I.

We also measured the levels of cytochrome c released from mitochondria into the cytosol. To measure the released cytochrome c into the cytosol, cells were lysed and fractionated into a cytosolic extract and mitochondrial pellet, as described in paper-II. Cytochrome c was measured by using a cytochrome c ELISA kit, as described in paper-II.

DNA fragmentation in growth plate tissue samples was detected by terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) immunohistochemistry according to instructions for the TdT-FragEL™

DNA fragmentation kit (Oncogene Research, Boston, MA). For details of digital automatic cell counting, please refer to paper-II, III and IV.

4.2.5 Analysis of Mitochondrial Membrane Potential (Paper-II)

To investigate the intrinsic/mitochondrial-mediated apoptotic signaling in Dexamethasone treated chondrocytes, we measured the loss of the mitochondrial membrane potential by using a sensitive fluorescent probe, tetramethylrhodamine ethyl ester (TMRE; Molecular Probes). Proliferative chondrocytes treated or untreated with dexamethasone were allowed to incubate with TMRE (100 nM) for 60 minutes at 37°C prior to the analysis on ELISA reader. In bortezomib studies (Paper-IV), MMP was measured using a FACSCalibur flow cytometer. Unfixed stem-like/resting C5.18, chondrocytes were treated with/without bortezomib, and TMRE-fluorescence was detected in live cells as determined by forward-scatter and side-scatter criteria. Data were analyzed in FLOW JO (version 6.4.7, Ashland, OR). As a negative control, we used PBS without TMRE. For details, please refer to paper-II and IV.

4.2.6 Western blot/Immunoprecipitation (Paper I-IV)

To study the regulation of different pro- and anti-apoptotic proteins such as Bax, p53, AIF, caspase-8, 9, and 3, PARP, p53, MDM-2 and Bcl-2, we used Western blotting. In paper-III, our western blot data showed that proteasome inhibition up-regulates AIF and that AIF acts as a target protein of Ub. To detect the binding of Ub with AIF, we performed immunoprecipitation. Briefly, cells treated/untreated with proteasome inhibitor MG262 were lysed in a radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Roche Diagnostics GmbH) and 1 mmol/L of phenylmethylsulfonyl fluoride. After this, supernatants were incubated with the AIF antibody (Santa Cruz) at 4°C for 2 hr followed by the addition of protein G-Sepharose CL-4B (Amersham Bioscience). After overnight incubation, the resulting immunocomplexes were subjected to SDS-PAGE.

4.2.7 Immunohistochemistry/Immunocytochemistry (Paper-II, III, IV)

In mouse/rat/human growth plate cartilage and fetal rat metatarsal bones:

To examine the up-regulation of p53, AIF, and Bax, we used growth plate cartilage from rats and mice. Human growth plate biopsies were also used to detect Bax up-regulation. Furthermore, fetal rat metatarsal bones cultured for 12 days with/without Dexa were stained with anti-Bax antibody. For immunohistochemistry of Bax, we used a specific anti-Bax antibody (clone 6A7) that detects Bax with conformational changes. In growth plate sections obtained from rat tibia, we also used HSP60 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to label the mitochondria. The slides were counterstained with DAPI for 15 min, and the resulting fluorescent signals were detected by fluorescence microscopy.

In cells:

To investigate whether Dexa treatment in chondrocytes induces translocation of Bax and the release of cytochrome c from mitochondria, we performed immunocytochemistry. HCS-2/8 chondrocytes were grown on glass coverslips and exposed to Dexa or IGF-1 for the indicated time. Cells were also labeled with MitoTracker® (to label the mitochondria) before fixing in 4% paraformaldehyde. Please refer to paper-II for detailed protocol.

4.2.8 Proteasome Activity, Serum IGF-I, Growth Plate morphometry, Alcian blue staining (Paper-II, III)

Serum levels of insulin-like growth factor-I were measured using a commercial RIA kit (Media Diagnostics, according to the instructions provided in the kit). We also verified proteasome inhibition by looking at proteasome activity in the blood, as reported in paper-III. In each tissue sample, at least 15 measurements of growth plate height were taken, and column density was determined as the number of chondrocyte columns per millimeter of the growth plate. All measurements were done by a person blinded to the experimental details. Nodule formation *in vitro* (paper-III), a chondrocyte differentiation marker (C5.18 chondrocytes, early

differentiated and differentiated/late differentiated cells), was confirmed with 5% Alcian blue staining.

5 RESULTS AND DISCUSSION

5.1 GC-INDUCED BONE GROWTH IMPAIRMENT AND APOPTOSIS IN CHONDROCYTES (PAPER-I, II)

Dexamethasone induces apoptosis in chondrocytes by activating caspases and suppressing Akt-PI3K signaling pathway (***Paper-I)***

Growth suppressive effects due to short-term use of GCs are temporary, and catch-up growth is seen soon after termination of the treatment. Long-term use of GCs, however, can have permanent growth suppressive effects ; Magiakou, Mastorakos et al. 1994). We have previously shown, along with others, that apoptosis of growth plate chondrocytes is increased in GC-induced growth retardation (Chrysis, Ritzen et al. 2003), although decreased cell proliferation and differentiation are other contributing factors reported for this effect. The fact that levels of apoptosis detected in growth plate cartilage is always very low does not mean that apoptosis is not associated with observed growth retardation of bones. It is possible that chondrocytes die at different stages of proliferation/differentiation, as chondrocytes in the growth plate are already passing through different stages of proliferation/differentiation. However, the limitation of the TUNEL assay is that it detects DNA fragmentation at one time point, and the only cells that are detected are those that have already undergone apoptosis and show signs of DNA fragmentation. This would mean that cells in early stages of apoptosis, such as during the activation of caspase-8, 9, or 3, will not be detected by TUNEL assay. In addition, it is also possible that other forms of cell death, such as autophagy, may co-exist alongside with apoptosis as it has recently been reported that GCs induce autophagy (Laane, Tamm et al. 2009) (Grander, Kharaziha et al. 2009).

Since we previously reported that GC-induced growth retardation is associated with increased apoptosis of chondrocytes (Chrysis, Ritzen et al. 2003), some cells in proliferative phase have been found to be positive with TUNEL assay. Based on these findings, we proposed that unnecessary/excessive apoptosis in proliferative chondrocytes can affect growth plate potential. Furthermore, there was no evidence suggesting that GC treatment of chondrocytes in the proliferative stage makes them more vulnerable to undergo apoptosis or which mechanism(s) may be involved.

Therefore, we decided to investigate the effects of GCs *in vitro* in chondrocytes only under the proliferation phase. To address this, we used HCS-2/8 chondrocytic cell line and challenged them to dexamethasone only under proliferation phase. In these experiments, we added IGF-I as a survival factor. The data obtained from these experiments show that dexamethasone not only significantly decreased chondrocytes viability and proliferation but also induced apoptosis in a dose-dependent manner **(Figure 5 A-B).**

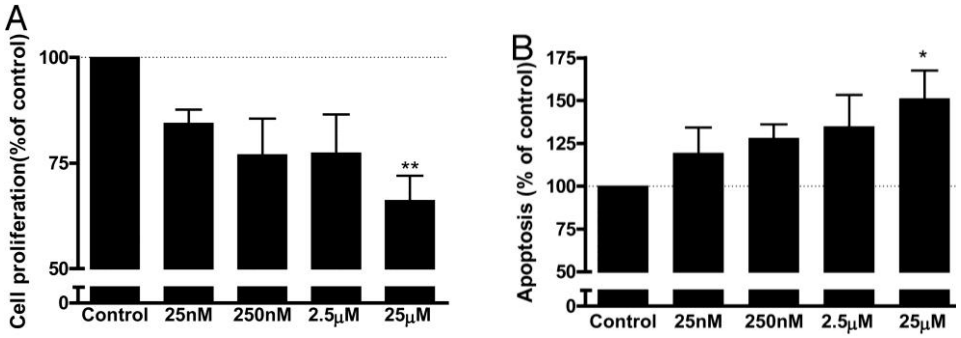

Figure. 5 Dose-response effects of Dexa (72 hr) on cell viability/proliferation (A) and apoptosis (B). Dexa had a clear dose-dependent inhibitory effect on cell viability (A) and a dose-dependent pro-apoptotic effect (B). *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

The observed apoptosis in chondrocytes appeared to be caspase-dependent, as inhibition of caspase-8 and 9 prevented these cells from undergoing apoptosis. Furthermore, caspase-8 was activated earlier than caspase-9 **(Figure 6A-B).** However, addition of IGF-I to dexamethasone groups rescued proliferative chondrocytes both in terms of proliferation and apoptosis but not to the control level.

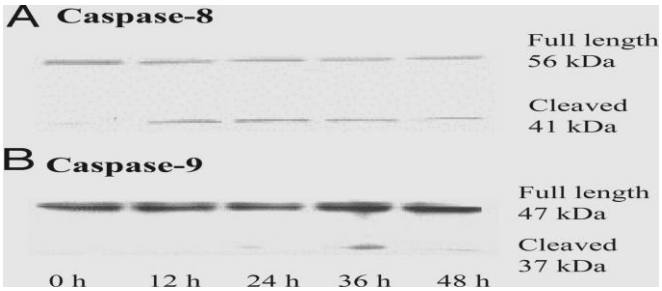

Figure 6. To investigate caspase cascade signaling, HCS 2/8 cells were cultured with 25 μM Dexa for 12, 24, 36, and 48 hr. The control was untreated cells at time point 0. Western blots were performed at all time points for caspase-8 (A) and caspase-9 (B). Cleaved caspase-8 was detected as early as after 12 hr, whereas active caspase-9 was detected after 36 hr.

The failure of IGF-I to rescue these proliferative chondrocytes suggested that activation of pro-survival signaling by increased phosphorylation of PI3-Akt is not

sufficient to protect chondrocytes from apoptosis. We found that inhibitory effects of dexamethasone on Akt phosphorylation were due to increased p85 inhibitory subunit, whereas rescuing effects of IGF-I was not completely dependent on Akt-PI3K. Interestingly, caspase-9 activation further suggested a strong role of mitochondrial-mediated apoptosis.

Absence of the pro-apoptotic protein Bax protects from glucocorticoid-induced bone growth impairment ***(Paper-II)***

In these studies, we have further characterized apoptosis induced by dexamethasone in chondrocytes *in vitro* and *in vivo*. Our data show that dexamethasone increases pro-apoptotic protein Bax levels in growth plate chondrocytes. Indeed, Bax has previously been reported to be regulated by phosphorylation of serine184 in an Akt-dependent manner. The phosphorylation inhibits the effects of Bax on the mitochondria by maintaining the protein in the cytoplasm (Gardai, Hildeman et al. 2004), whereas in paper-I, we reported that Dexa suppresses Akt phosphorylation in chondrocytes. In addition, in the same studies we reported that the observation of caspase-9 activation (Chrysis, Zaman et al. 2005) also suggested that Dexa may target mitochondria to trigger apoptosis in proliferative chondrocytes. Interestingly, it has also been reported that Bax deficiency increases bone mineral density (BMD) and cartilage production in fractured bone (Rundle, Wang et al. 2008) suggesting multiple roles of Bax. Altogether, these signaling events led us to further investigate the role of the Bcl-2 family member of proteins Bax in Dexa-induced chondrocyte apoptosis and impaired bone growth. In fact, it is still unknown whether GC treatment in proliferative chondrocytes can induce mitochondrial dysfunction leading to apoptosis by altering expression of the Bcl-2 family of proteins. A better understanding of these mechanisms may have important implications in the prevention of growth retardation caused by glucocorticoid treatment during childhood.

To investigate intrinsic apoptotic signaling pathways, the human chondrocytic cell line HCS-2/8 was treated with Dexa (25 µmol/L), and mitochondrial membrane potential (MPT) was measured. We found that MPT was significantly suppressed after 24 and 48 hr, and cytochrome c was also found to be released in the cytosol after Dexa exposure. Furthermore, both up-regulation of Bax

and conformational changes were verified by using western blots and immunocytochemistry **(Figure. 7A-B).**

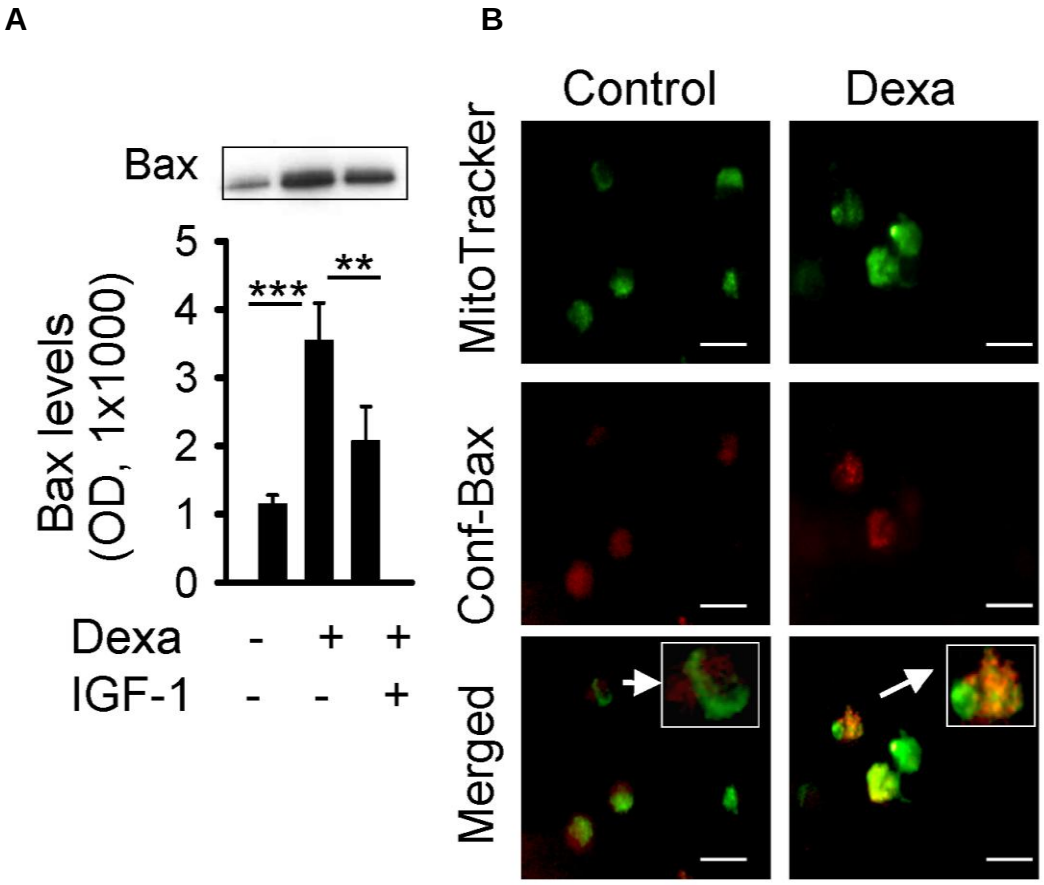

Figure 7. Dexa up-regulates Bax expression and induces conformational changes in Bax. (A) HCS proliferative chondrocytes treated with Dexa were analyzed for the expression of the Bax protein. (B) Conformational changes in Bax and translocation to mitochondria. Immunocytochemistry of HCS proliferative chondrocytes treated or untreated with Dexa. Cells were analyzed for conformational changes in Bax using a specific antibody that only detects conformationally altered Bax. Chondrocytes were labeled with Bax antibody (red) and MitoTracker® (green). Bax was mainly found in the mitochondria of Dexa-treated cells, as appeared by the yellow-orange staining that is due to the merged red and green fluorescence.

To further confirm if up-regulation of Bax is a critical step in Dexa-induced chondrocyte apoptosis, Bax expression was suppressed using specific siRNAs. Our data show that in non-silenced, control cells, Dexa increased apoptosis by 59%. However, transfection with Bax siRNAs completely rescued chondrocytes from Dexa-induced apoptosis. Based on the assumption that Bax plays a key role in apoptosis in chondrocytes upon treatment with Dexa, one would predict that Bax-deficient mice are

resistant to Dexa-induced growth retardation. To validate this hypothesis, Bax-deficient mice were treated with a clinically relevant dose of Dexa (2 mg/kg body weight) for 28 days and X-rays were taken on a weekly basis to measure longitudinal bone growth (femur and tibia). In wild-type mice, Dexa treatment caused a significant reduction in femur growth. This effect was much less pronounced in Bax-deficient mice **(Figure 8A)**. Dexa treatment significantly increased the number of TUNEL positive cells in the growth plate chondrocytes in wild-type mice. However, no difference was observed in the apoptosis levels between animals treated with Dexa or vehicle in Bax-deficient mice **(Figure 8B)**.

These data suggest that loss of Bax protects chondrocytes from undesired Dexa-induced apoptosis and bone growth retardation. In addition, loss of Bax appears to have a positive impact on bone formation, as we detected intense Alcian Blue/van Gieson staining in Bax-deficient growth plates.

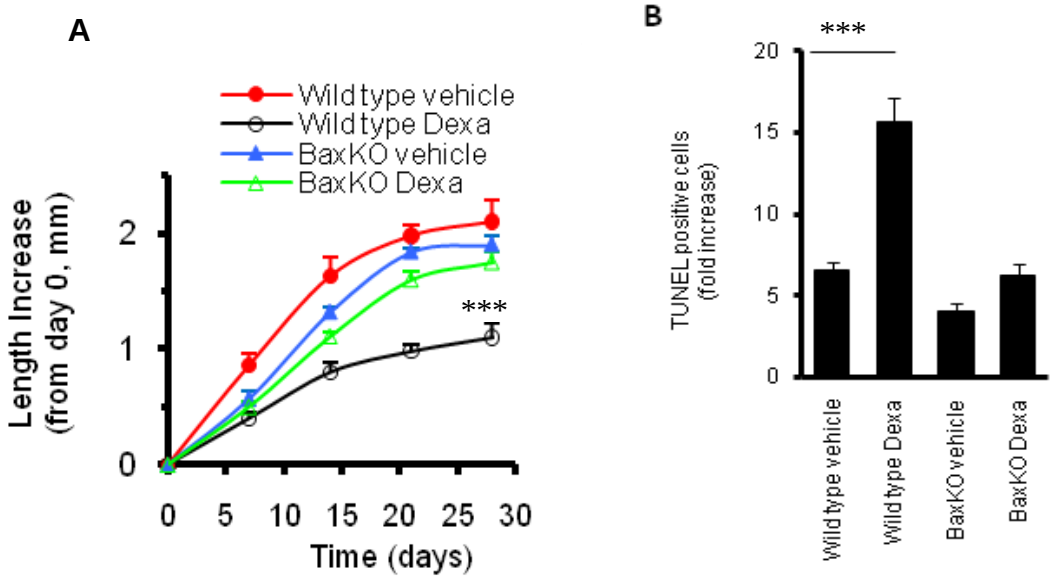

Figure 8. (A) Bax-deficient and wild type female mice were treated with Dexa or saline for 28 days. X-rays were taken on days 0, 7, 14, 21, and 28, and left femur lengths were measured (*n* =5, Bax KO; n = 5, vehicle). (B) TUNEL assays for the detection of DNA fragmentation in growth plates of Bax-deficient and wild-type mice treated with Dexa or vehicle for 28 days. **p<0.01, ***p<0.001.

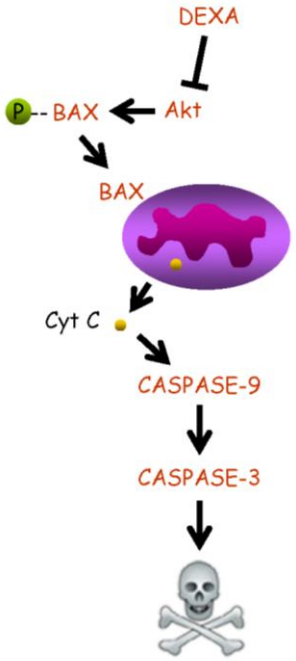

Figure 9. Schematic figure depicting the putative pathway of Dexa-induced apoptosis in proliferative chondrocytes. Bax is known to be regulated by phosphorylation in an Akt-dependent manner. Our previous studies have shown that Dexa suppresses Akt-phosphorylation. The present study demonstrates that Dexa treatment leads to Bax activation and translocation to mitochondria with cytochrome c release and apoptosis induction of chondrocytes.

In conclusion, we have used an array of model systems to show that Dexa induces apoptosis **(Figure 9)** and premature loss of growth plate chondrocytes that result in impaired longitudinal bone growth. Importantly, we also demonstrated a key role of Bax in the pathogenesis of Dexa-induced bone growth retardation.

5.2 EFFECTS OF PROTEASOME INHIBITION ON BONE GROWTH (PAPER-III, IV)

Proteasome inhibition up-regulates p53 and apoptosis inducing factor in chondrocytes causing severe growth retardation in mice ***(Paper-III)***

In these studies, we have investigated the effects of proteasome inhibition on longitudinal bone growth. Novel pro-apoptotic anti-cancer agents (proteasome inhibitors), such as bortezomib, are now emerging in clinical practice; they have already been approved for the treatment of multiple myeloma and are currently under clinical trials for childhood cancers (Blaney, Bernstein et al. 2004). However, effects of proteasome inhibitors in fast-growing individuals are unknown.

To address this question, we have used an array of model systems, including cell cultures, organ cultures of fetal rat metatarsal bones, and normal and transgenic mice models. In these studies, we show that that systemic administration of PIs such as MG262 and bortezomib induces growth retardation. Systemic administration **(Figure 10)** of proteasome inhibitors specifically impairs the ubiquitin/proteasome system in growth plate chondrocytes. Importantly, young mice displayed severe growth retardation during treatment and 45 days after the cessation of treatment with clinically relevant amounts of MG262 (0.2 μmol/kg body weight/injection) or bortezomib (1.0 mg/kg body weight/injection). An impaired ubiquitin/proteasome system was accompanied by the induction of apoptosis of stem-like and proliferative chondrocytes in the growth plate cartilage.

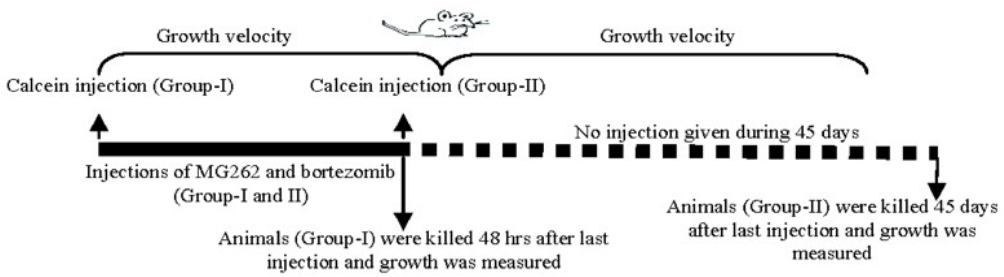

Figure 10. Diagram detailing the injection scheme of proteasome inhibitor MG262 administered to the animals.

Investigating the mechanism(s) of growth retardation caused by proteasome inhibition, we observed that the resting zone of chondrocytes was severely affected and consisted of very few cells in MG262-treated animals **(Figure 11A)**. Caspase inhibitory experiments showed that proteasome inhibition was triggering both caspase-dependent and independent apoptosis in chondrocytes. We also found that p53 levels were increased in the growth plate cartilage of mice treated with MG262 **(Figure 11B)**. In addition, AIF, a regulator of caspase independent apoptosis, also appeared to be highly up-regulated both *in vitro* and *in vivo* **(Figure 11C)**.

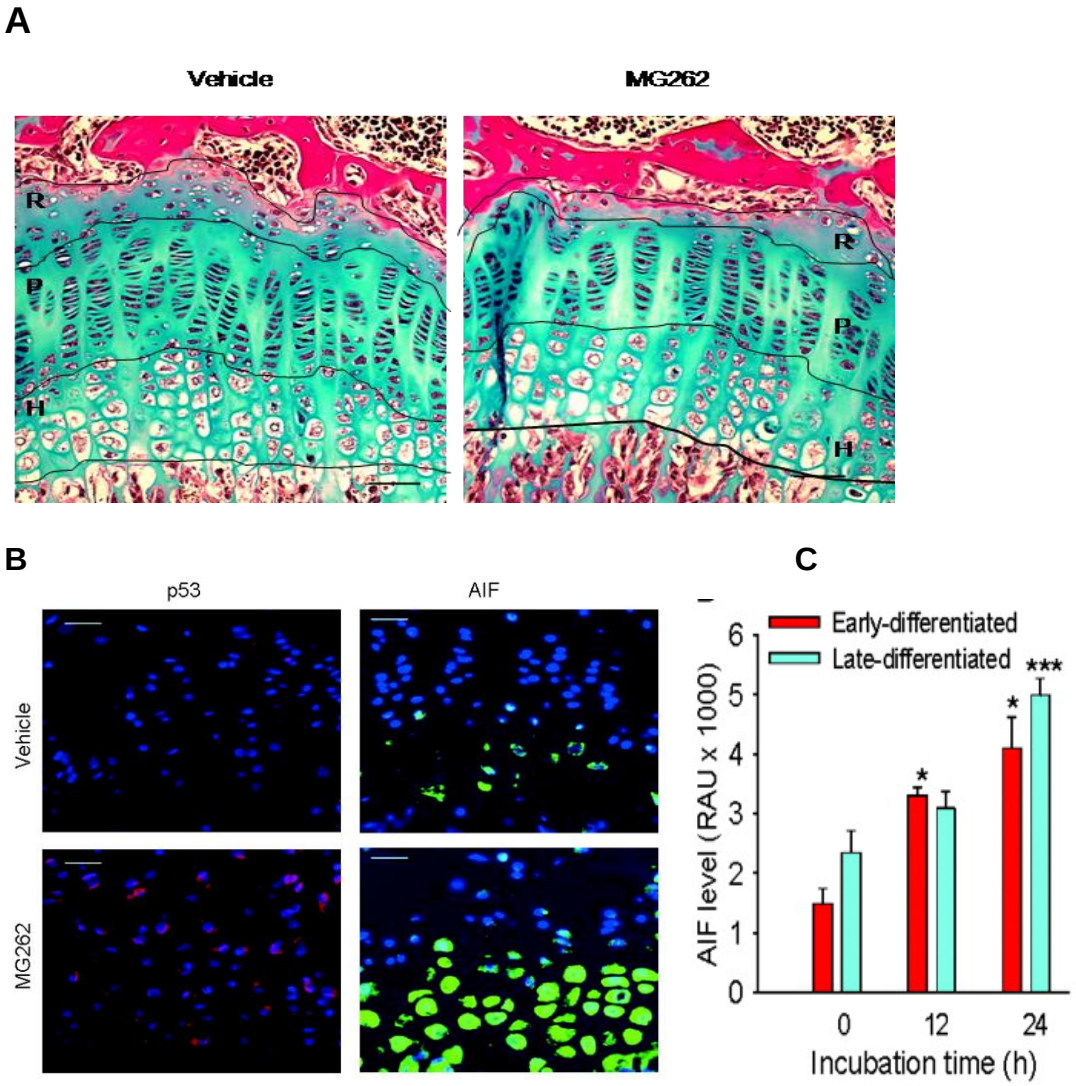

Figure 11. (A) *A* representative micrographs of growth plate (tibia) showing different zones affected by proteasome inhibition: resting (*R*), proliferative (*P*), and hypertrophic (*H*) zones. (B) Immunohistochemistry of growth plate cartilage showing up-regulation of pro-apoptotic proteins p53 and AIF. (C) Quantitative analysis of western blots in C5.18 chondrocytes, during early differentiated (comparable to resting chondrocytes) or late differentiated phase (comparable to proliferative chondrocytes).

Suppression of p53 expression using siRNAs resulted in a 35% decrease in apoptosis in MG262-treated cells, indicating that p53 is involved in the induction of apoptosis within the growth plate cartilage. Furthermore, in support of a role for AIF-mediated apoptotic cell death, suppression of AIF with sequence-specific siRNAs decreased the apoptosis of rat chondrocytes by 41%. Taken together, these data show that bone growth is impaired *in vitro* and in young mice due to the deleterious effects of proteasome inhibition on chondrocytes.

In conclusion, in these pre-clinical models we report that the systemic administration of proteasome inhibitors impairs chondrogenesis by triggering apoptosis and decreases chondrocyte proliferation, thus causing growth retardation. The fact that proteasome inhibition may selectively target essential cell populations in the growth plate causing significant growth failure suggests that children treated with PIs may be at risk for permanent side effects limiting bone growth potential.

*Bortezomib induces apoptosis in stem cell-like chondrocytes and impairs longitudinal bone growth **(Paper-IV)***

In paper-III, we showed that proteasome inhibition causes growth retardation in young mice. While we and others have experimentally demonstrated that preclinical proteasome inhibitors can induce chondrocyte apoptosis by regulating p53 and AIF (Wu and De Luca 2006; Zaman, Menendez-Benito et al. 2007; Zaman, Fadeel et al. 2008), the mechanism by which the clinically-used proteasome inhibitor, bortezomib, affects growth plate chondrocytes remains to be elucidated. Therefore, in these studies we have focused on effects of bortezomib in growth plate cartilage. In addition, we also have tested the cytotoxicity of bortezomib in human growth plate biopsies. First, we treated fetal rat metatarsal bones to observe the direct effects of bortezomib on longitudinal bone growth. Fetal rat metatarsal bones were cultured for 12 days in the presence of bortezomib **(Figure 12)**. The data obtained from these experiments indicate that bortezomib induces growth retardation in bones.

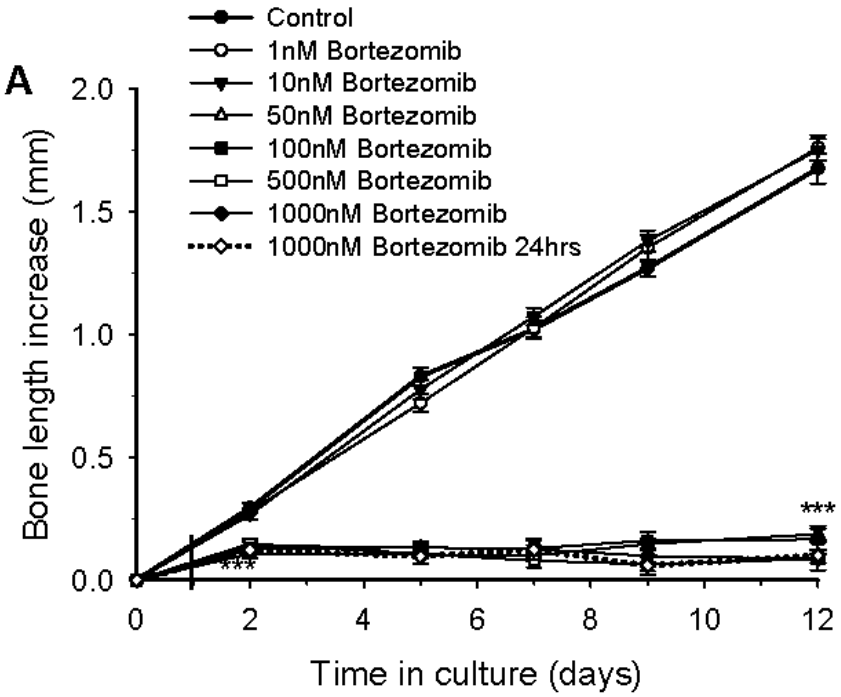

Figure 12. As shown in this figure, it is clear that even very low doses of bortezomib treatment permanently impairs longitudinal growth of cultured fetal rat metatarsal bones cultured *ex vivo* for 12 days (1-1000 nM). For one of the groups, bortezomib (1000 nM 2h hr) treatment was restricted to the first 24 hr of culture, and thereafter bones were transferred to control medium.

Interestingly, bortezomib treatment for only 24 hr was enough to permanently inhibit the bone growth **(Figure 12)**. These data suggest that toxic effects of bortezomib were more severe to an essential cell population responsible for longitudinal bone growth. To identify this essential cell population, we analyzed bortezomib-treated bones with TUNEL assay. Bortezomib significantly increased DNA fragmentation in chondrocyte apoptosis **(Figure 13A)**, with mostly resting/stem-like cells stained. Metatarsal bones were also stained with Alcian Blue/van Gieson to detect changes of matrix components, such as GAGs and collagens. Indeed, bortezomib decreased the levels of matrix components.

The sensitivity of stem-like/resting chondrocytes was further verified by cell viability (MTT-assay) analyzed in the rat C5.18 cell-line. The cells were treated for 24 or 48 hr with bortezomib (0-100 nM), which resulted in a dose- and time-dependent decrease in cell viability of mostly resting/stem-like chondrocytes. In contrast, proliferative and hypertrophic chondrocytes were more resistant to bortezomib treatment.

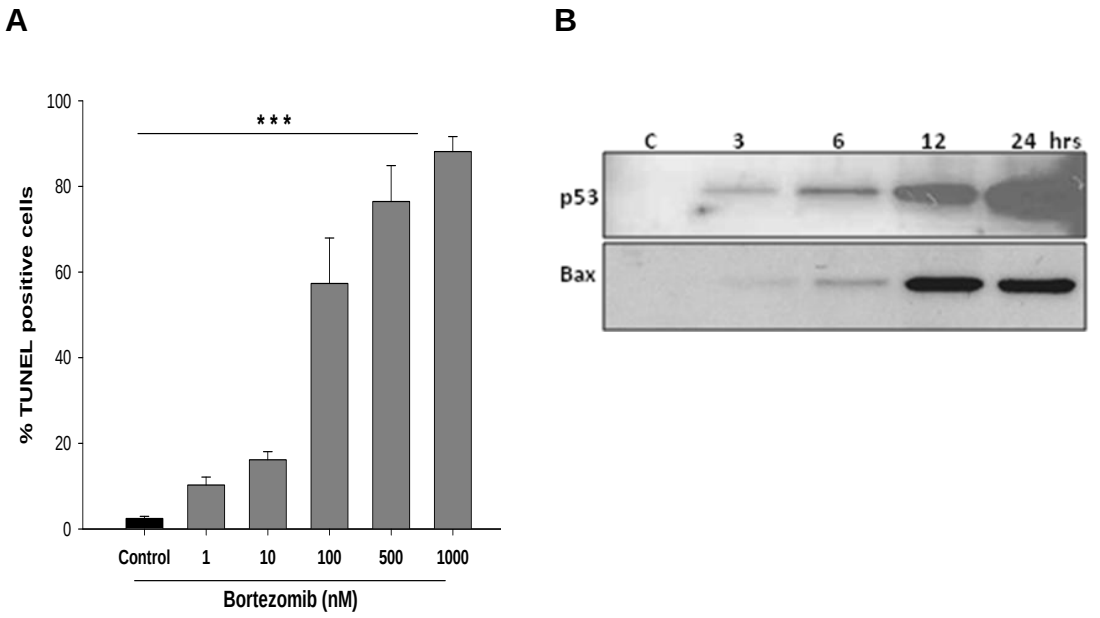

Figure 13. (A) Metatarsal bones were analyzed by TUNEL assay for DNA fragmentation. Data represent means ± SEM. ***p<0.001 versus the corresponding control. (B) Changes in protein expression induced by bortezomib (1000 nM) in rat resting/stem-like chondrocytes (C5.18 cells) for 0 to 24 hrs and analyzed for regulation of p53 and Bax.

In an attempt to delineate possible mechanisms regulating bortezomib-induced apoptosis, protein expression profiles (using western blot) of several pro-and anti-apoptotic proteins were determined in resting/stem-like C5.18 chondrocytes exposed to bortezomib for 3, 6, 12, or 24 hrs **(Figure 13B)**. We found that bortezomib increased levels of p53 and Bax as early as 3 hrs after treatment **(Fig 13B)** suggesting key roles of these two pro-apoptotic proteins in bortezomib-induced apoptosis in resting chondrocytes.

In summary, we show that in clinically relevant doses, bortezomib exerts cytotoxic effects on resting/stem-like chondrocytes, resulting in impairment of linear bone growth. In the observed intrinsic apoptotic pathway p53 and/or Bax appear to play a key role. Based on these data, we suggest that further investigation of the cytoprotective strategies to minimize undesired toxic effects on linear bone growth when PIs are given to children is warranted.

6. CONCLUDING REMARKS

1. We provide evidence that dexamethasone induces apoptosis in proliferative chondrocytes by activating caspase-8, 9, and 3 and suppressing the Akt-PI3K signaling pathway. These findings suggest that caspase-9 activation in the intrinsic apoptotic pathway is also activated in proliferative chondrocytes.

2. We report a novel finding that the pro-apoptotic protein Bax is a molecular target to prevent the growth retardation/impairment caused by dexamethasone. Dexamethasone-induced Bax activation was validated with an array of model systems. Silencing of Bax with siRNA rescued chondrocytes from dexamethasone-induced apoptosis, and Bax-deficient young mice were resistant to dexamethasone-induced growth retardation. Interestingly, bone formation was also increased in Bax-deficient mice compared to wild-type mice.

3. Investigating the effects of PIs on bone growth, we show for the first time that systemic administration of PIs specifically target stem-like and proliferative chondrocytes in the growth plate causing growth retardation in young mice. PIs triggered chondrocyte apoptosis that was mediated at least in part through p53 and AIF.

4. We report that the proteasome inhibitor bortezomib, in a clinically relevant dose, targets resting/stem-like chondrocytes resulting in permanent impairment of linear bone growth *in vitro*. In the observed effect on the intrinsic apoptotic pathway, p53 and/or Bax appear to play key roles.

7. FUTURE PERSPECTIVES

In this thesis work, we hypothesized that premature loss of chondrocytes contribute to growth failure caused by the use of GCs. Investigating the mechanism(s) of dexamethasone-induced apoptosis in proliferative chondrocytes, we suggest a new molecular target for the prevention of growth impairment caused by GCs. Our present finding that Bax-deficient mice are resistant to growth retardation caused by Dexa is of considerable clinical relevance because in tissue specimens obtained from rheumatoid arthritis patients, higher levels of Bax have been reported than in healthy controls (Hilbers, Hansen et al. 2003). In addition, strong Bax staining was also found in chondrocytes at sites of cartilage degradation (Hilbers, Hansen et al. 2003). The fact that Bax appears to play a critical role in suppression of bone growth suggests that small molecules/peptides targeted toward Bax could be effective in decreasing Bax activation in patients with diseases associated with up-regulated Bax. Moreover, they could potentially be useful when treating growing individuals with GCs. Indeed, small molecule-based therapeutics targeted toward other Bcl-2 family members are currently being explored in the context of cancer treatment (Oltersdorf, Elmore et al. 2005).

Whether PIs such as bortezomib exert effects on bone growth and cartilage in children remains unknown. However, our data from these pre-clinical studies show for the first time that PIs severely impair longitudinal bone growth, an effect associated with increased apoptosis (p53, AIF and Bax) of stem-like chondrocytes. Our findings show that clinically relevant doses of PIs, including bortezomib and MG262, induce growth retardation and thus emphasize the need to scrutinize carefully the clinical use of PIs in children, as essential cell populations in bone may be selectively targeted, causing impaired bone growth. We suggest that a combination of a pro-apoptotic drug and a cytoprotective agent may be a feasible and advantageous approach. For instance, PIs are known to induce both p53-dependent and independent apoptosis. The combination of p53 inhibitor and PIs in p53-mutant cancer cells should not interfere with PIs ability to induce apoptosis, whereas the normal chondrocytes will be protected because apoptosis in these cells is mediated via p53. However, a comprehensive characterization of PIs can help us identify new molecular targets for the prevention of undesired side effects.

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